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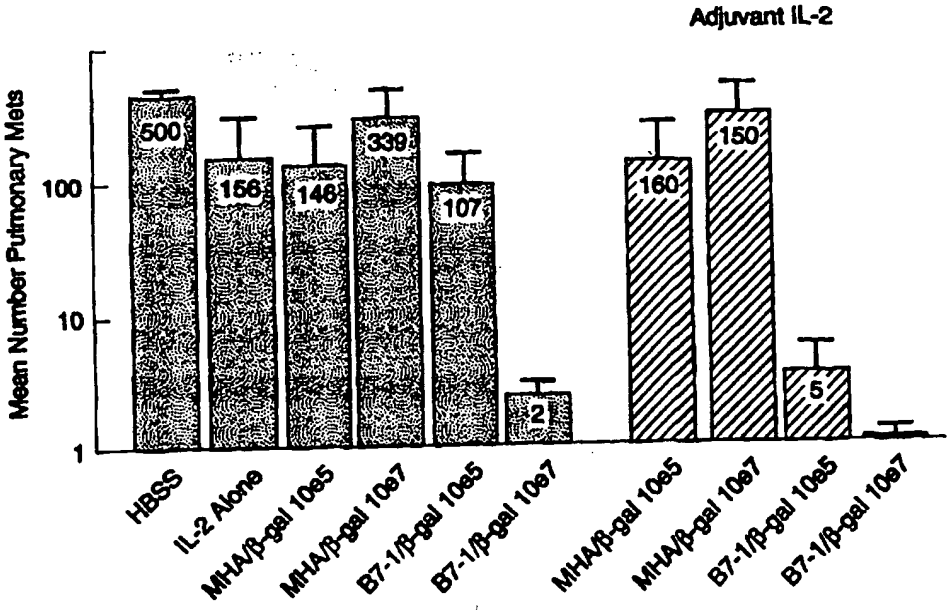
(54) Title: ENHANCED IMMUNE RESPONSE BY INTRODUCTION OF CYTOKINE GENE AND/OR COSTIMULATORY MOLECULE B7 GENE IN A RECOMBINANT VIRUS EXPRESSING SYSTEM



(57) Abstract

The present invention is a recombinant virus which has incorporated into its genome or portion thereof a gene encoding an antigen at a disease causing agent in combination with an immunostimulatory molecule for the purpose of stimulating an immune response against the disease causing agent. Methods of treatment of diseases such as cancer and diseases caused by pathogenic microorganisms are provided using the recombinant virus.

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ENHANCED IMMUNE RESPONSE BY INTRODUCTION OF CYTOKINE GENE AND/OR COSTIMULATORY MOLECULE B7 GENE IN A RECOMBINANT VIRUS EXPRESSING SYSTEM

FIELD OF THE INVENTION

The present invention relates to recombinant viral vector vaccines for the prevention or treatment of pathogenic diseases and cancer. More particularly, it relates to recombinant viral vector vaccines comprising a genes encoding an antigens and a gene(s) encoding an immunostimulatory molecule(s).

BACKGROUND OF THE INVENTION

The five year survival of patients with metastatic melanoma is less than 2% in most reported series. Combination chemotherapy can induce objective regressions of melanoma but this treatment is rarely, if ever, curative. Tumor infiltrating lymphocytes (TIL) have been identified in patients with melanoma that appear to recognize unique cancer antigens in an MHC restricted fashion. (Rosenberg, S.A. J. Clin. Oncol. 10:180-199, 1993; Topalian, S.C. et al Tumor-infiltrating lymphocytes: Evidence for specific immune reactions against growing cancers in mice and humans. In: De Vita, Hellman and Rosenberg (eds) Important Advances in Oncology, Philadelphia, J.B. Lippincott Co., 1990, pp 19-41, Schwartzentruber et al J. Immunol 146:3674-3681, 1991). The adoptive transfer of TIL can mediate regression in 35 to 40% of patients with advanced melanoma and studies are underway to attempt to generate more potent TIL by genetic modification of TIL or by immunization with preparations containing tumor associated antigens. Rosenberg, J. Clin. Oncol. 10:180-199, 1993; Rosenberg, S.A. J. Am. Med. Assoc 268:2416-2419, 1992.

A large number of studies in experimental animals have demonstrated that the cellular rather than the humoral arm of the immune response plays the major role in the elimination of murine tumors. Wunderlich, J.R. et al.

Principles of tumor immunity: In: DeVita et al (eds) Biologic Therapy of Cancer, Philadelphia: J.B. Lippincott Co. 1991, pp 3-21. Much of this evidence was derived from studies in which the adoptive transfer of T lymphocytes from immune animals could transfer resistance to tumor challenge or in some experiments the actual elimination of established cancer. Thus, most strategies for the immunization of patients with cancer have been directed at stimulating strong T cell immune reactions against tumor associated antigens.

Most attempts at active immunization against cancer antigens have involved whole tumor cells or tumor cell fragments, though it would be most desirable to immunize specifically against unique tumor antigens that distinguish malignant from normal cells. The molecular nature of the tumor associated antigens recognized by T lymphocytes is poorly understood. In contrast to antibodies that recognize epitopes on intact proteins, T cells recognize short peptide fragments (8-18 amino acids) that are presented on cell surface class I or II major histocompatibility (MHC) molecules and it is likely that tumor associated antigens are presented and recognized by T cells in this fashion.

A number of genes have been identified that encode melanoma tumor antigens recognized by TIL in the context of the HLA-A2 class I molecule. Kawakami, T. et al Proc. Nat'l Acad. Sci. 91:3515-3519, 1994; Kawakami, Y. et al J. Exp. Med. 180:347-352, 1994; Kawakami et al Cancer Res. 54:3124-3126, 1994. These antigens appear to be the most clinically relevant antigens responsible for mediating tumor regression in patients with advanced melanoma since the TIL used to identify these antigens were capable of mediating in vivo antitumor regression. Two such antigens, which appear to be present in virtually all fresh and cultured melanomas, have been called MART-1 (Melanoma Antigen Recognized by T Cells - 1) and gp100.

The genes encoding both of the peptides have been cloned and sequenced. The MART-1 gene encodes a 118 amino acid protein of 13 kD. The gp100 gene encodes a protein identical to that recognized by monoclonal antibody HMB-45. With the exception of melanocytes and retina no normal tissues express this antigen and no expression of these gene products has been seen on cancers other than melanoma. Both antigens, therefore, appear to be melanocyte lineage specific.

The MART-1 antigen was expressed on all nine tissue culture lines tested (that were established from melanomas in the Surgery Branch, NCI) and on all fresh melanomas tested. Studies by others showed that the MART-1 (also called Melan-A) antigen was expressed on 26 of 26 fresh melanomas. Coulie, P.G. et al J. Exp. Med. 180:35-42, 1992. The gp100 antigen is also widely expressed in melanomas. In one study, reactivity with antibody HMB-45 (reactive with gp100) was present on 100% of non-spindle cell type melanomas and on 62 of 67 total melanomas. Wick, M.R. et al J. Cutan. Pathol. 4:201-207, 1988. In another study, 32 of 35 melanomas studied (91%) expressed gp100 (Ordonez, N.G. et al Am. J. Clin. Pathol. 4:385-390, 1988) and in a third study of 60 of 62 (97%) melanomas expressed gp100 (Gown, A.M. et al Am. J. Pathol. 123:195-203, 1986).

Of 14 separate TIL cells that were raised in the Surgery Branch, NCI from different HLA-A2 individuals, 13 of 14 recognized MART-1 and 4 of 14 recognized the gp100 antigen. Because TIL cells that recognize these determinants have been shown to be capable of mediating cancer regression in vivo, it appears that these antigens are involved in cancer regression.

Another gene coding for a human tumor specific antigen on a human melanoma was cloned by Van der Bruggen et al (Science 254:1643-1647, 1991). This antigen is coded for by a gene called MAGE-1 which spans five

kilobases. A 2,419 base pair coding sequence produces a predicted protein product of 26 kD. The MAGE antigen is HLA-A1 restricted and the nine amino acid fragment that represents the A1 restricted immunodominant peptide has been defined as Glu-Ala-Asp-Pro-Thr-Gly-His-Ser-Tyr. This nine amino acid peptide is encoded by the third exon of the MAGE gene. Transfection of a 500 base pair fragment of this gene can confer recognition by a MAGE specific CTL clone. Incubation of an EBV cell line with the immunodominant peptide can confer sensitivity to lysis by a MAGE-1 specific CTL clone. MAGE-1 does not appear to be expressed in normal cells with the possible exception of testis, but is expressed on approximately half of metastatic melanomas, about 20% of breast cancers as well as other selected types of cancer.

The identification of an immunodominant peptide that represents a unique tumor antigen has opened new possibilities for immunization against cancer. Substantial evidence exists in animal models that immunization with immunodominant viral peptides can induce viral specific CTL that can confer protection against viral infection. Although pure peptide alone is ineffective in stimulating T cell responses, peptides emulsified in adjuvants or complexed with lipids have been shown to prime mice against challenge with fresh virus and can induce virus specific CTL that protect mice against lethal viral inocula (Kast, W.M. et al Proc. Nat'l Acad. Sci. U.S.A. 88:2283-2287, 1991; Deres, K. et al Nature 342:561-564, 1989; Gao, X.M. et al J. Immunol. 147:3268-3273, 1991; Aichele, P. J. Exp. Med. 171:1815-1820, 1990; Collins, D.S. et al J. Immunol. 148:3336-3341, 1992). Immunization of mice against splenocytes coated with Listeria monocytogenes peptide epitopes also results in the generation of Listeria specific CTL which can be expanded in culture. Adoptive transfer of these CTL can protect mice against lethal bacterial challenge (Harty,

J.T. et al J. Exp. Med. 175:1531-1538, 1992). Peptides representing antigenic epitopes of HIV gp120 and gp160 emulsified in complete Freund's adjuvant can also prime specific CTL responses (Takahashi, H. et al Proc. Nat'l Acad. Sci. U.S.A. 85:3105-3109, 1988; Hart, M.K. et al Proc. Nat'l Acad. Sci. U.S.A. 88:9448-9452, 1991).

While immunization with peptides in adjuvants or complexed with lipids gives rise to T cell responses in mice, the reactions are rarely strong enough to induce T reactive cells in primary splenocytes. The detection of sensitized lymphocytes almost invariably requires secondary in vitro stimulation.

The expression of the B7 gene family has been shown to be an important mechanism of antitumor responses in both mice and humans. It is now becoming apparent that at least two signals are required for activation of naive T-cells by antigen bearing target cells: an antigen specific signal, delivered through the T-cell receptor, and an antigen independent or costimulatory signal leading to lymphokine products (Hellstrom, K.E. et al. Annals NY Acad. Sci. 690:225-230, 1993). Two important costimulatory molecules are B7-1, which is the ligand for T-cell surface antigens CD28 and CTLA4 (Schwartz, R.H. Cell 71:1065-1068, 1992; Chen, L. et al. Cell 71:1093-1102, 1992; Freeman, G.J. et al. J. Immunol 143:2714-2722, 1989; Freeman, G.J. et al. J. Exp. Med. 174:625-631, 1991), and B7-2, an alternative ligand for CTLA4 (Freeman, G.J. et al. Science 262:813-960, 1995). To date, both murine B7-1 and B7-2 (Freeman, G.J. et al. J. Exp. Med. 174:625-631, 1991; Freeman, G.J. et al. Science 262:813-960, 1995) and human B7-1 and B7-2 have been described (Freeman, G.J. et al. J. Immunol 143:2714-2722, 1989; Freeman, G.J. et al Science 262:909-911, 1993). It is unclear at this time whether the costimulatory signals provided by B7-1 and B7-2 are functionally distinct or redundant mechanisms for T-cell activation (Hathcock, K.S.

et al. J. Exp. Med. 180:631-640, 1994). Most murine and human tumors do not express B7-1 or B7-2, implying that even when a tumor expresses a potential rejection antigen, it is unlikely to activate antitumor T-cell responses (Hellstrom, K.E. et al Annals. N.Y. Acad. Sci. 690:225-230, 1993); Hellstrom, I. Annals. N.Y. Acad. Sci. 690:24-31, 1993). In essence, anergy may result from only one signal being received by the T-cell (Hellstrom, K.e. et al. Annals. N.Y. Acad. Sci. 690:225-230, 1993. Transfection of B7 into melanoma cells was found to induce the rejection of a murine melanoma in vivo (Townsend, S.E. et al Science 259:368-370, 1993).

Vaccinia viruses have been extensively used in humans and the use of a vaccina based vaccine against smallpox has led to the worldwide eradication of this disease (reviewed in reference Moss, B. Science 252:1662-1667, 1991). Vaccinia viruses have the advantages of low cost, heat stability and a simple method of administration. Attempts have been made to develop vaccinia virus vectors for the prevention of other diseases.

Vaccina virus is a member of the pox virus family of cytoplasmic DNA viruses. DNA recombination occurs during replication of pox viruses and this has been used to insert DNA into the viral genome. Recombinant vaccina virus expression vectors have been extensively described. These vectors can confer cellular immunity against a variety of foreign gene products and can protect against infectious diseases in several animal models. Recombinant vaccina viruses have been used in human clinical trials as well. Cooney et al immunized 35 healthy HIV seronegative males with a recombinant vaccinia virus expressing the gp160 envelope gene of HIV (Cooney, E.. The Lancet 337:567-572, 1991). Graham et al randomized 36 volunteers to receive either recombinant vaccinia virus containing the gp160 HIV envelope protein or control vaccinia virus (Graham, B.S. et al J. Infect. Dis. 166:244-252, 1992).

Phase I studies using recombinant vaccinia virus have begun in patients with metastatic melanoma using a recombinant virus expressing the p97 melanoma antigen (Estin, C.D. et al Proc. Nat'l Acad. Sci. 85:1052-1056, 1988) and a trial to use recombinant vaccinia virus expressing the human carcinoembryonic antigen in patients with advanced colorectal carcinoma is about to begin (Schlom, J. personal communication). In these trials, vaccinia virus is administered by intradermal scarification and side effects have been minimal including local skin irritation, lymphadenopathy and transient flu-like symptoms.

Fowlpox viruses are members of the pox virus family (avipox virus genes). Fowlpox virus will only replicate in avian cells and cannot replicate in human cells. It is a cytoplasmic virus that does not integrate into the host genome but is capable of expression of a large number of recombinant genes in eukaryotic cells.

Recombinant fowlpox virus expressing rabies glycoprotein has been used to protect mice, cats and dogs against live rabies virus challenge. Immunization of chickens and turkeys with a recombinant fowlpox expressing the influenza HA antigen protected against a lethal challenge with influenza virus (Taylor, J. et al Vaccine 6:504-508, 1988). Canarypox virus, another member of the avipox genus similar to fowlpox, was safely administered subcutaneously to 25 normal human volunteers at doses up to 10" infectious doses (Cadox, M. et al The Lancet 339:1429-1432, 1992). In a recent trial sponsored by the NIAID (Protocol 012A: A Phase I safety and immunogenicity trial of live recombinant canarypox-gp160 MN (ALVAC VCP125 HIV-1gp160MN0 in HIV-1 uninfected adults) patients received recombinant canarypox virus containing the HIV gp160 gene by intramuscular injection at doses up to 10 pfu with little to no toxicity (personal communication, P. Fast, NIAID).

Fowlpox virus thus represents an attractive vehicle for immunization since it can stimulate both humoral and cellular immunity, it can be economically produced in high titer (10^9 pfu/ml) and yet its inability to productively infect human cells substantially increases the safety of its use, compared to replicating viruses such as vaccina view, especially in immunocompromised hosts.

Another considerable advantage of fowlpox virus is that there is apparently little or no cross-reactivity with vaccinia virus and thus previously vaccinated humans will not have pre-existing immune reactivity to fowlpox virus proteins.

SUMMARY OF THE INVENTION

The present invention is a recombinant virus comprising a viral genome or portion thereof, one or more nucleic acid sequences encoding one or more antigens of a disease causing agent and one or more nucleic acid sequences encoding one or more immunostimulatory molecules.

The present invention is also a composition comprising a recombinant virus comprising a viral genome or portion thereof and one or more nucleic acid sequences encoding one or more antigens of a disease causing agent and optionally an exogenous immunostimulatory molecule, chemotherapeutic drug, antibiotic, antifungal drug, antiviral drug, or combination thereof.

Another aspect of the present invention is a composition comprising a recombinant virus comprising a viral genome or portion thereof, one or more nucleic acid sequences encoding one or more antigens of a disease causing agent and one or more nucleic acid sequences encoding one or more immunostimulatory molecules and optionally an exogenous immunostimulatory molecule, chemotherapeutic drug, antibiotic, antifungal drug, antiviral drug, or combination thereof.

Another aspect of the present invention is to provide

a therapeutic composition and a method of treating or preventing a disease in a mammal comprising administering to the mammal an effective amount of a recombinant virus and optionally an exogenous immunostimulatory or immunomodulator, chemotherapeutic drug antibiotic, antifungal drug, antiviral molecule, the amount effective in preventing or ameliorating the disease.

It is also an object of the present invention to provide a method of making an immune enhancing recombinant virus against a disease causing agent comprising inserting a nucleic acid sequence encoding one or more antigens or immunodominant epitopes thereof and a nucleic acid sequence encoding one or more immunostimulatory molecules into the genome or portion thereof of a recombinant virus.

It is also an object of the present invention to provide an immune enhancing recombinant virus against a disease causing agent comprising an immune enhancing recombinant virus comprising a nucleic acid sequence encoding one or more antigens or immunodominant epitopes thereof and a nucleic acid sequence encoding one or more immunostimulatory molecules.

It is also an object of the present invention to provide a vaccine against a disease causing agent comprising a recombinant virus containing a nucleic acid sequence encoding one or more antigens or immunodominant epitopes thereof and a nucleic acid sequence encoding one or more immunostimulatory molecules. The vaccine of the present invention is able to prevent or inhibit infection or disease caused by the disease causing agent.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other objects, feature and many of the attendant advantages of the invention will be better understood upon a reading of the detailed description of the invention.

Figure 1 shows β-Galactosidase production after BSC1 cell infection with various recombinant vaccinia virus

constructs after 24 hr (■) or 36 hr (□) incubation.

Figure 2 shows the primary response of mice at Day 6 after injection I.V. with various recombinant vaccinia virus constructs as follows: GM-CSFrVV (□); IL-2rVV (●); TNFαrVV (▼); IFNγrVV(▽); VJS6 (O). Effector: Target cells ratios are plotted versus % specific ^{51}Cr release by targets.

Figure 3 shows the results from secondary cultures of mice at Day 6 after injection I.V. with various recombinant vaccinia virus constructs as follows: GM-CSFrVV (□); IL-2rVV (●); TNFαrVV (▼); IFNγrVV(▽); VJS6 (O). Effector: Target cells ratios are plotted versus % specific ^{51}Cr release by targets.

Figure 4 shows the results from secondary cultures of mice at Day 14 after injection I.V. with various recombinant vaccinia virus constructs as follows: GM-CSFrVV (□); IL-2rVV (●); TNFαrVV (▼); IFNγrVV(▽); VJS6 (O). Effector: Target cells ratios are plotted versus % specific ^{51}Cr release by targets.

Figure 5 shows the primary response of mice against vaccinia-infected CT26 tumor cells at Day 6 after injection S.C. with various recombinant vaccinia virus constructs as follows: GM-CSFrVV (●); IL-2rVV (□); TNFαrVV (▽); IFNγrVV(▼); VJS6 (■). Effector: Target cells ratios are plotted versus % specific ^{51}Cr release by targets.

Figure 6 shows the titration of lytic units 30% from a primary response of mice against vaccinia-infected CT26 tumor cells at Day 6 after injection S.C. with various recombinant vaccinia virus constructs as follows: GM-CSFrVV (●); IL-2rVV (□); TNFαrVV (▽); IFNγrVV(▼); VJS6 (■). Effector cells per well is plotted versus % specific ^{51}Cr release from CT26VAC target cells.

Figure 7 shows the results from secondary cultures of mice at Day 14 after injection S.C. with various recombinant vaccinia virus constructs as follows: GM-

CSFrVV (□); IL-2rVV (●); TNFαrVV (▾); IFNγrVV(▿); VJS6 (○). Effector: target cells ratios are plotted versus % specific ^{51}Cr release by targets.

Figure 8 shows active anti-tumor immunotherapy in mice using exogenous IL-2 together with recombinant vaccinia virus expressing a tumor associated antigen. Treatment groups included HBSS (—); rVV+BG (— -); rVV-BG + HD IL-2 (100,000 I.U., I.P., BID x 3 days) (▬); rVV+BG + HD IL-2 (100,000 I.U., I.P., BID x 3 days) (- - - -); rVV + BG + LD IL-2 (15,000 I.U., BID x 3 days (▬). Survival time (days) is plotted versus proportion of surviving mice.

Figure 9 shows survival data in an active-tumor immunotherapy model in mice with an established non-transduced tumor, CT26. GM-CSFrVV (▲); IL-2rVV (■); TNFαrVV (▮); IFNγrVV(◆); VJS6 (▾); HBSS (●). Days after tumor injection is plotted versus % surviving mice.

Figure 10 shows survival data in an active tumor immunotherapy model in mice with established β-gal expressing tumor, CT 26.C25. GM-CSFrVV (▲); IL-2rVV (■); TNFαrVV (▮); IFNγrVV(◆); VJS6 (▾); HBSS (●). Days after tumor injection is plotted versus % surviving mice.

Figure 11 shows active treatment of established pulmonary metastases using various recombinant vaccinia constructs. The average number of pulmonary metastases is plotted versus recombinant vaccinia vector used for treatment. HB2m = Human Beta-2-microglobulin rVV, Ld = murine H-2L^d MCH Class I molecule rVV, NA = Neuramimidase rVV, MVA = Ankara - attenuated vaccinia virus, Kb = murine H-2K^b MCH Class I molecule rVV.

Figure 12 shows active treatment of established pulmonary metastases using various recombinant vaccinia constructs. The average number of pulmonary metastases is plotted versus recombinant vaccinia vector used for treatment. ICAM-1 = Intracellular adhesion molecule-1 rVV, D^d = murine H2-D^d MCH Class I molecule rVV, K^d =

murine H-2 K^d MHC Class I molecule rVV, L^d = H-2 L^d MHC Class I molecule rVV; K^b = H-2K^b MHC Class I molecule rVV; HLA-A2.1 = Human MHC Class I molecule rVV, $h\beta_2m$ = Human Beta-2-microglobulin rVV, NA = neuramimidase rVV, GM-CSF = granulocyte, monocyte colony stimulatory factor rVV, IFNα = interferon gamma rVV, TNFα = tumor necrosis factor alpha rVV, MVA = Ankara - attenuated vaccinia virus, HBSS = Hank's Balanced Salt Solution.

Figure 13 shows a Western blot analysis of recombinant vaccinia virus expressed murine B7-1. BS-C-1 cells were infected with 1, v. MCB7-1; 2, v. MCMHA and extracts were prepared 20 hours post infection. A murine specific B7-1 hamster monoclonal antibody was used to detect expression of recombinant proteins. ^{125}I protein A (0.1 μCi/ml) and autoradiography were used to identify bound antibody. Protein sizes were estimated using ^{14}C molecular weight markers (MW).

Figure 14 shows the results of non-irradiated BALB/c mice (5/group) which were injected intravenously with 5 x 10^5 tumor cells of CT26.WT or CT26.C25 on day #0. On day #3 mice were immunized intravenously with different recombinant vaccinia viruses (10^7 PFUs). Mice were randomized, and euthanized on day #12. Lung were harvested and stained, and pulmonary metastases were enumerated in a blinded fashion. All mice injected with CT26.WT had 500 metastases (data not shown). This graph represents a compilation of data drawn from several separate experiments. The individual rVV were involved in the following number of experiments: v. IL2/β-gal -- five; v. MCB7-1/β-gal -- six; v. D^d/β-gal -- one; v. K^d/β-gal -- one, v. L^d/β-gal -- one, v. K^b/β-gal -- one, v. HLA.A2.1/β-gal -- one, v. $m\beta_2m$/β-gal -- one, v. $h\beta_2m$/β-gal -- one, v. GM-CSF/β-gal -- four, v. IFN-γ/β-gal -- four, v. TNF-α/β-gal -- four; v. JS6 -- greater than five, v. MVA/β-gal --- one.

Figure 15 shows the results of non-irradiated BALB/c

mice (10/group) which were injected intravenously with HBSS, v. MCB7-1/β-gal, or v. MCMHA/β-gal. Twenty-one days later mice were challenged intravenously with 5 x 10^5 tumor cells CT26.C25 or CT26.WT. Mice were randomized, and euthanized on day #12. Lung were harvested and stained, and pulmonary metastases were enumerated in a blinded fashion. Duplicate experiments confirmed these results.

Figure 16 shows the results of non-irradiated BALB/c mice (10/group) which were injected with 10^5 or 10^7 PFUs of v. MCB7-1/β-gal, v. MCMHA/β-gal, or HBSS on day #0. Twenty-one days later a splenectomy was performed on all immunized mice. 2 x 10^7 splenocytes from designated groups of immunized mice were adoptively transferred to similar mice (5/group) injected intravenously three days earlier with 5 x 10^5 tumor cells of CT26.C25 or CT26.WT. Designated groups of mice were treated with IL-2 (100,000 IU i.p. BID x 3 days). Mice were randomized and euthanized nine days later. Lung were harvested and stained, and pulmonary metastases were enumerated in a blinded fashion. All mice inoculated with CT26.WT had large tumor burdens (data not shown). Duplicate experiments confirmed these results.

Figure 17 shows the results of non-irradiated BALB/c mice (5/group) which were injected intravenously with 5 x 10^5 CT26.WT or CT26.C25 tumor cells on day #0. On day #3 mice were immunized intravenously with 10^7 PFUs of v. MCB7-1/β-gal, v. MCMHA/β-gal, v. MCB7-1/NP or HBSS. On day# 3 - 6, designated groups of mice were treated with IL-2 (100,000 IU i.p. BID x 3 days). Mice were randomized, and euthanized on day #12. Lung were harvested and stained, and pulmonary metastases were enumerated in a blinded fashion. All mice inoculated with CT26.WT had large tumor burdens (data not shown). Four separate experiments confirmed these results.

Figure 18 shows the results of non-irradiated BALB/c

mice (5/group) which were injected intravenously with 5 x 10^5 CT26.WT or CT26.C25 tumor cells on day #0. On day #6 mice were immunized intravenously with 10^7 PFUs of v. MCB7-1/β-gal, v. MCMHA/β-gal, v. MCB7-1/NP or HBSS. On day# 6 - 9, designated groups of mice were treated with IL-2 (100,000 IU i.p. BID x 3 days). Mice were randomized, and euthanized on day #12. Lung were harvested and stained, and pulmonary metastases were enumerated in a blinded fashion. All mice inoculated with CT26.WT had large tumor burdens (data not shown). Duplicate experiments confirmed these results.

Figure 19 shows the results of non-irradiated BALB/c mice (10/group) which were injected intravenously with 5 x 10^5 CT26.WT or CT26.C25 tumor cells on day #0. On day #3 mice were immunized intravenously with 10^7 PFUs of v. MCB7-1/β-gal, v. MCMHA/β-gal, v. MCB7-1/NP or HBSS. On day# 3 - 6, designated groups of mice were treated with IL-2 (100,000 IU i.p. BID x 3 days). All mice inoculated with CT26.WT were dead by day # 41 (data not shown). Survival was followed daily and events recorded as deaths.

Figure 20 shows the results of non-irradiated BALB/c mice (5/group) which were injected intravenously with 5 x 10^5 CT26.WT or CT26.C25 tumor cells on day #0. On day #3 mice were immunized intravenously with 10^7 PFUs of v. MCB7-1/β-gal, v. MCB7-2/β-gal, v. MCB7-1/B7-2/β-gal, v. MCMHA/B7-2/β-gal, v. MCMHA/β-gal, v. MCB7-1/NP, or HBSS. On day# 3 - 6, designated groups of mice were treated with IL-2 (100,000 IU i.p. BID x 3 days). Mice were randomized, and euthanized on day #12. Lung were harvested and stained, and pulmonary metastases were enumerated in a blinded fashion. All mice inoculated with CT26.WT had large tumor burdens (data not shown). Duplicate experiments confirmed these results.

Figures 21a through 21f show a FACS analysis of splenocytes isolated from normal non-depleted (Figs. 21a

and 21b), T_{CD8+}-depleted (Figs. 21c and 21d), or T_{CD4+}-depleted (Figs. 21e and 21f) mice. Mice were given two i.v. injections of GK1.5 at 100 mg/ml or of empirically determined levels of 2.43 monoclonal antibodies 48 hours prior to receiving tumor challenge, and again 6 days later. Upper left hand panel in each square displays percent CD8+ cells, upper right panel shows percent CD4+CD8+ cells, lower right shows percent CD4+, and lower left shows scatter for all cells. Figure 21a, no depletion Using fluorescein isothiocyante-labeled anti-CD4 and phycoerythrin labeled anti-CD8 antibodies, FACS analysis was performed 1 day prior to immunization, and again at day 7 to verify depletion.

Figure 21g shows the mean number of pulmonary metastases of CD4- and CD8- immuno depleted mice vaccinated with V.B7-1/β-gal. Prior to tumor challenge non-irradiated BALB/c mice (5/group) were injected with anti-CD4 and anti-CD8 monoclonal antibodies (GKI.5 (anti-CD4) and 2.43 (anti-CD8). On day#0 mice were injected intravenously with 5 x 10^5 CT26.C25 tumor cells. On day #3 mice were immunized intravenously with 10^7 PFUs of v. MCB7-1/β-gal, v. MCMHA/β-gal, v. MCB7-1/NP or HBSS. On day #6, the mice were again injected with anti-CD4 and anti-CD8 monoclonal antibodies. Mice were randomized, and euthanized on day #12. Lung were harvested and stained, and pulmonary metastases were enumerated in a blinded fashion. FACS analysis was performed 1 day prior to immunization, and again at day 7 to verify depletion. Only data from v.B7-1/B-gal vaccination groups is shown. Duplicate experiments confirmed these results.

Figures 22a and 22b show active immunotherapy is enhanced when exogenous rIL-2 and rVV are given in concert. BALB/c mice (five per group) were challenged i.v. with 5 x 10^5 CT26.WT (Fig. 22a) or CT26.CL25 tumor cells (Fig. 22b). After 3 days they received a single i.v. injection of medium alone (HBSS) or medium containing

5 x 10^6 PFU of different TK$^-$ rVV either expressing (VJS6) or not expressing (V69) β-gal. Two different regimens of rIL-2 administration were started 12 h after rVV injection: high dose (HD, 100,000 Cetus U, twice a day, i.p. for 3 days) or low dose (LD, 15,000 Cetus U, twice a day, i.p. for 5 days). Mice were checked twice a day for survival.

Figures 23a and 23b show that exogenous rIL-2 enhances the function of rFPV. BALB/c mice (five per group) were inoculated i.v. with 5 x 10^5 CT26.WT (Fig. 23a) or CT26.CL25 tumor cells (Fig. 23b). On day 3 after tumor injection, they received a single i.v. injection of the following viruses: no virus (HBSS alone), 10^7 PFU of FPV.bg40k (rVPV), or FPV wild-type (FPVwt). rIL-2 (100,000 Cetus U, twice a day) was administered i.p. starting 12 h after FPV injection and continued for 3 days. Mice were checked twice a day for survival.

Figure 24 shows exogenous rIL-2 plus rVV is therapeutic in the more advanced 6-day tumor model. BALB/c, mice (five per group) were inoculated i.v., with 10^5CT26.CL25 tumor cells. Six days after tumor injection, they received the same treatments described in Figures 22a and 22b with the exception that only the highest dose of rIL-2 was administered. Mice were checked daily for survival. No prolongation of survival was obtained by the various treatments in mice bearing 6-day-old pulmonary metastases of CT26.WT tumor (data not shown).

Figures 25a and 25b show a drVV expressing IL-2, but not GM-CSF, IFN-γ, or TNF-α, significantly reduces the number of pulmonary metastases in a 3-day model. Five BALB/c mice per group were injected i.v. with 5 x 10^5 tumor cells of either CT26.WT or CT26.CL25 cell lines. Three days later they received a single i.v. injection of HBSS alone (none) or containing 5 x 10^6PFU/mouse of different rVV, as indicated. On day 12 post tumor challenge, lungs were harvested and pulmonary nodules were

enumerated in a blind fashion. An independent repeat of this experiment gave identical results.

Figures 26a and 26b show the function of a drVV expressing IL-2 is further enhanced when additional exogenous IL-2 is provided. BALB/c mice (five per group) were challenged i.v. with 5 x 10^5 CT26.WT (Fig. 26a) or CT26.CL25 tumor cells (Fig. 26b). After 3 days they received a single i.v. injection of plain medium (HBSS) or medium containing 5 x 10^6 PFU of rVV encoding β-gal alone (VJS6) or together with IL-2 (IL-2 rVV). Twelve hours after rVV, 100,000 rIL-2U were inoculated, according to the regimen described in Figs. 22a and 22b. Mice were checked twice a day for survival.

Figure 27 shows that expression of IL-2 by drVV enhances the antivaccinia CTL response. Two BALB/c mice were immunized with 5 x 10^6 PFU/mouse of different rVV. After 6 days the spleens were aseptically removed, mixed together, and tested directly in a 6-h ^{51}Cr release assay against CT26.WT tumor cell line, either infected (CT26 vaccinia) or noninfected (CT26.WT) during the isotope labeling with more than 10:1 moi of crude 19 VV preparation. Spontaneous release of target cells never exceeded 20%. E:T cell ratio was 100:1 and then 1:3 dilutions. Lytic units 30% (L.U. 30%) indicate the number of effector cells required to obtain 30% lysis of 10,000 target cells. L.U. 30% were normalized for the total number of cells recovered for each spleen and expressed as total L.U./spleen.

Figure 28 shows that the presence of tumor cells specifically enhance the CTL response elicited by IL-2 rVV in a dose-dependent manner. BALB/c mice were injected with HBSS alone or with varying doses of CT26.WT or CT26.CT25 as specified. After 3 days, mice were immunized with 5 x 10^6 PFU/mouse of either VJS6 or IL-2 rVV. On day 9 after tumor challenge (day 6 after vaccination) the primary cytotoxic response was evaluated in a 6-h ^{51}Cr

release assay against CT26.WT, CT26.WT pulsed with the β-gal L^d-restricted peptide (CT26T + peptide), CT26.CL25, and the irrelevant target cells E22 as shown. The effects of escalating doses of tumor on the generation of the primary cytotoxic response is shown for the immunization with IL-2rVV in nontumor-bearing mice (▲), or in mice bearing 5 x 10^4 CT26.CL25 (▲), 1 x 10^5 CT26.CL25 (●), 5 x 10^5 CT26.CL25 (O), or the highest dose, 5 x 10^5 of the parental (non-β-gal-expressing) CT26.WT cell line (□). An additional control is the non-IL-2-expressing VJS6 virus injected in mice bearing 5 x 10^5 CT26.CL25 (■).

Figure 29 shows the effects of recombinant IL-10 on the therapeutic effectiveness of immunization with the vaccinia virus, VJS6 + IL-1 (●); VJS6 alone (X).

Figure 30 shows the IL-10 adjuvant therapy on the therapeutic effectiveness of immunization with various concentrations of recombinant vaccinia virus encoding the model tumor antigen.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is a novel recombinant virus expressing an antigen(s) from a disease causing agent and an immunostimulatory molecule(s). The novel recombinant virus is capable of eliciting or upregulating an immune response in a mammal to T-dependent antigens for the purpose of preventing or treating a disease. The novel recombinant virus of the present invention is particularly important in upregulating cell-mediated immunity.

Cell-mediated immunity is crucial to resistant to diseases caused by cancer and pathogenic microorganism, particularly viruses and other intracellular microorganisms.

The recombinant virus has incorporated into its genome or portion thereof a gene encoding an antigen from a disease causing agent and one or more genes encoding one or more immunostimulatory molecules. A host cell infected

with the recombinant virus expresses both the antigen(s) from a disease causing agent and expresses the immunostimulatory molecule(s). The antigen may be expressed at the cell surface of the infected host cell. The immunostimulatory molecule may be expressed at the cell surface or may be actively secreted by the host cell.

The expression of both the antigen and the immunostimulatory molecule provides the necessary MHC restricted peptide to specific T cells and the appropriate signal to the T cell to aid in antigen recognition and proliferation or clonal expansion of antigen specific T cells. The overall result is an upregulation of the immune system. In a preferred embodiment the upregulation of the immune response is an increase in antigen specific cytotoxic lymphocytes which are able to kill or inhibit the growth of a disease causing agent or a cell infected with a disease causing agent.

In one embodiment, the recombinant virus comprises the virus genome or portions thereof, the nucleic acid sequence encoding an antigen from a pathogenic microorganism and one or more nucleic acid sequences encoding one or more immunostimulatory molecules.

In another embodiment, the recombinant virus comprises the virus genome or portions thereof, the nucleic acid sequence encoding a tumor associated antigen, and one or more nucleic acid sequences encoding one or more immunostimulatory molecules.

In one embodiment the recombinant viruses have been constructed and co-express model tumor antigens together with cytokines (TNF-α, IFN-γ, GM-CSF, IL-10 and IL-2), restriction elements (class 1 α-chains and β_2m), and co-stimulatory and accessory molecules (B7-1, B7-2 and ICAM-1 and the like) alone and in a variety of combinations. Simultaneous production of an immunostimulatory molecule and the model TAA at the site of virus replication/infection (in any case, the site of TAA

production) enhances the generation of specific effectors. Dependent upon the specific immunostimulatory molecules, different mechanisms might be responsible for the enhanced immunogenicity: augmentation of help signal (IL-2), recruitment of professional APC (GM-CSF), increase in CTL frequency (IL-2), effect on antigen processing pathway and MHC expression (IFNγ and TNFα) and the like. The co-expression of a model antigen together with at least one immunostimulatory molecule is effective in an active immunotherapy model.

The present invention also encompasses a recombinant virus comprising the virus genome or portion thereof, the nucleic acid sequence encoding the antigen of interest and more than one nucleic acid sequences encoding more than one immunostimulatory molecule for the added benefit of upregulating an immune response against the antigen.

In some cases it may be beneficial to make a recombinant virus comprising more than one antigen of interest for the purpose of having a multivalent vaccine. For example, the recombinant virus may comprise the virus genome or portions thereof, the nucleic acid sequence encoding GP120 (from HIV), the nucleic acid sequence encoding Hep B surface antigen and one or more immunostimulatory molecules.

In one embodiment, the recombinant virus comprises the vaccinia virus genome or portions thereof, the nucleic acid sequence encoding MART-1 and the nucleic acid sequence encoding the immunostimulatory molecule, B 7.1 alone or in combination with the nucleic acid sequence encoding the immunostimulatory molecule, B7.2.

In another embodiment, the recombinant virus comprises the fowlpox virus genome or portions thereof, the nucleic acid sequence encoding MART-1, the nucleic acid sequence encoding MAGE-1, and the nucleic acid sequence encoding the immunostimulatory molecule, IL-2, alone or in combination with the nucleic acid sequence

encoding the immunostimulatory molecule, B7.1. In a specific embodiment the recombinant virus is McB7-1, McB71-1/NP, McB7-1β-gal, McB7-1/ova, McB7-1/PIA, McB7-1/B7-2, McMHA, McMHA/NP, McMHA/β-gal, McMHA/ova, McMHA/PIA, McMHA/B7-2, McB7-2, McB7-2/β-gal, IL-2(β-gal)rVV, GM-CSF(β-gal)rVV, IFNγ(β-gal)rVV, IL-10(β-gal) rVV, and TNFα(β-gal)rVV.

The insertion of costimulatory molecules and/or cytokine genes in recombinant vaccinia virus (rVV genome containing the model TAA) is beneficial in treatment of established metastases.

The present invention encompassed a composition comprising a recombinant virus containing a viral genome or portion thereof, one or more nucleic acid sequences encoding one or more antigens from one or more disease causing agents and one or more nucleic acid sequences encoding one or more immunostimulatory molecules. The compositions of the present invention may also comprise an exogenous immunostimulatory molecule or combinations of immunostimulatory molecules and/or may comprise a chemotherpeutic drug, antibiotic, antifungal drug, antiviral drug and the like and combinations thereof.

In one embodiment, the composition contains a recombinant virus which has incorporated into its genome or portion thereof a gene encoding a tumor associated antigen and exogenous IL-2. In another embodiment, the composition contains a recombinant virus which as incorporated into its genome or portion thereof a gene encoding a tumor associated antigen in combination with exogenous IL-10.

Virus Vectors

Virus that may be used in the present invention are those in which a portion of the genome can be deleted to introduce new genes without destroying infectivity of the virus. The virus vector of the present invention is a nonpathogenic virus. In one embodiment the virus vector

has a tropism for a specific cell type in the mammal. In another embodiment, the virus vector of the present invention is able to infect professional antigen presenting cells such as dendritic cells and macrophages. In yet another embodiment of the present invention, the virus vector is able to infect any cell in the mammal. The virus vector may also infect tumor cells.

The virus of the present invention include but is not limited to Poxvirus such as vaccinia virus, fowlpox virus and a highly attenuated vaccinia virus (Ankara or (MVA)), retrovirus, adenovirus, baculovirus and the like.

The vaccinia virus genome is known in the art. It is composed of a HIND F13L region, TK region, and an HA region. The recombinant vaccinia virus has been used in the art to incorporate an exogenous gene for expression of the exogenous gene product (Perkus et al. Science 229:981-984, 1985; Kaufman et al. Int. J. Cancer 48:900-907, 1991; Moss Science 252:1662, 1991).

A general strategy for construction of vaccinia virus expression vectors have been described (Smith and Moss Bio Techniques Nov/Dec, p. 306-312, 1984; U.S. Patent No. 4,738,846). Briefly, the first step in formation of recombinant viruses expressing heterologous DNA is the construction of a chimeric gene containing a vaccinia promoter fused to the protein coding sequences of the foreign gene. The chimeric gene is assembled in a plasmid vector and engineered so that the transcriptional start site of the promoter is positioned close to the translational initiation codon of the foreign gene. Importantly, additional ATG triplets between the transcriptional and translational start sites should be eliminated so that fusion polypeptides or incorrect reading frames are avoided. Generally, the promoters are included in DNA fragments 200-300 base pairs in length, but smaller fragments have also proved functional. After their assembly in plasmid vectors, the chimeric genes are

inserted into the virus genome by homologous recombination *in vivo*. This method is necessary since the great size of vaccinia DNA makes *in vitro* construction of recombinant molecules impractical. To facilitate homologous recombination, the chimeric gene is first flanked by vaccinia DNA taken from a non-essential region of the virus genome. The resulting plasmid, called a recombination vector, is transfected into vaccinia virus-infected cells, whereupon homologous recombination results in site-specific insertion of the chimeric gene into the virus genome. Recombinant genomes are replicated and packaged into infectious progeny virus within the infected cells. The nature of the flanking DNA is important since this determines the site of insertion. Only nonessential regions can be used and several of these have now been identified. A commonly used locus is the vaccinia TK gene since recombinant viruses are consequently TK.

A gene encoding an antigen of a disease causing agent may be incorporated into the HIND F13L region or alternatively incorporated into the TK region of recombinant vaccinia virus vector. Likewise, a gene encoding an immunostimulatory molecule may be incorporated into the HIND F13L region or the TK region of recombinant vaccinia virus vector.

Sutter and Moss (Proc. Nat'l. Acad. Sci U.S.A. 89:10847-10851, 1992) and Sutter et al. (Virology 1994) disclose the construction and use as a vector, the non-replicating recombinant Ankara virus (MVA, modified vaccinia Ankara) which may be used as a viral vector in the present invention.

Baxby and Paoletti (Vaccine 10:8-9, 1992) disclose the construction and use as a vector, of the non-replicating poxvirus, including canarypox virus, fowlpox virus and other avian species which may be used as a viral vector in the present invention.

Expression vectors suitable for use in the present

invention comprise at least one expression control element operationally linked to the nucleic acid sequence. The expression control elements are inserted in the vector to control and regulate the expression of the nucleic acid sequence. Examples of expression control elements includes, but is not limited to, lac system, operator and promoter regions of phage lambda, yeast promoters and promoters derived from polyoma, adenovirus, retrovirus or SV40. Additional preferred or required operational elements include, but are not limited to, leader sequence, termination codons, polyadenylation signals and any other sequences necessary or preferred for the appropriate transcription and subsequent translation of the nucleic acid sequence in the host system. It will be understood by one skilled in the art the correct combination of required or preferred expression control elements will depend on the host system chosen. It will further be understood that the expression vector should contain additional elements necessary for the transfer and subsequent replication of the expression vector containing the nucleic acid sequence in the host system. Examples of such elements include, but are not limited to, origins of replication and selectable markers. It will further be understood by one skilled in the art that such vectors are easily constructed using conventional methods (Ausubel et al., (1987) in "Current Protocols in Molecular Biology", John Wiley and Sons, New York, New York) or commercially available.

Disease Causing Agents

The recombinant virus of the present invention is effective in treating or preventing disease caused by disease causing agents or a disease state. Each disease causing agent or disease state has associated with it an antigen or immunodominant epitope on the antigen which is crucial in immune recognition and ultimate elimination or control of the disease causing agent in a mammal,

sometimes referred to in the art as a protective antigen. The mammalian immune system must come in contact with the antigen or immunodominant epitope on the antigen in order to mount a humoral and/or cellular immune response against the associated disease causing agent.

The recombinant virus of the present invention comprises the one or more nucleic acid sequences encoding one or more isolated antigens or immunodominant epitopes on the antigens and one or more nucleic acid sequences encoding one or more immunostimulatory molecules for the purpose of enhancing immune response against the disease causing agent.

Such disease causing agents include but are not limited to cancer and pathogenic microorganisms or mammals. Mammals include but are not limited to humans, primates, rats, mice, guinea pigs, rabbits, horses, cows, sheep, pigs, goats and the like. Cancers of mammals which may be treated using the recombinant virus of the present invention include but are not limited to melanoma, metastases, adenocarcinoma, thymoma, lymphoma, sarcoma, lung cancer, liver cancer, colon cancer, non-Hodgkins lymphoma, Hodgkins lymphoma, leukemias, uterine cancer, breast cancer, prostate cancer, ovarian cancer, cervical cancer, bladder cancer, kidney cancer, pancreatic cancer and the like.

The term melanoma includes, but is not limited to, melanomas, metastatic melanomas, melanomas derived from either melanocytes or melanocytes related nevus cells, melanocarcinomas, melanoepitheliomas, melanosarcomas, melanoma in situ, superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma, acral lentiginous melanoma, invasive melanoma or familial atypical mole and melanoma (FAM-M) syndrome. Such melanomas in mammals may be caused by, chromosomal abnormalities, degenerative growth and developmental disorders, mitogenic agents, ultraviolet radiation (UV), viral infections,

inappropriate tissue expression of a gene, alterations in expression of a gene, and presentation on a cell, or carcinogenic agents.

The aforementioned cancers can be assessed or treated by methods described in the present application. In the case of cancer, a gene encoding an antigen associated with the cancer is incorporated into the recombinant virus genome or portion thereof along with a gene encoding one or more immunostimulatory molecules. The antigen associated with the cancer may be expressed on the surface of a cancer cell or may be an internal antigen. In one embodiment the antigen associated with the cancer is a tumor associated antigen (TAA) or portion thereof. Examples of TAA that may be used in the present invention include but are not limited to melanoma TAAs which include but are not limited to MART-1 (Kawakami et al. J. Exp. Med. 180:347-352, 1994), MAGE-1, MAGE-3, GP-100, (Kawakami et al. Proc. Nat'l. Acad. Sci. U.S.A. 91:6458-6462, 1994), CEA, TRP-1, P-15, and tyrosinase (Brichard et al. J. Exp. Med. 178:489, 1993) and the like.

The nucleotide sequence of the MAGE-3 gene is disclosed in Gaugler et al. J. Exp. Med. 179:921-930, 1994. MAGE-3 is expressed on many tumors of several types, such as melanoma, head and neck squamous cell carcinomas, lung carcinoma and breast carcinoma but not in normal tissues except for testes. The approximately 1.6 Kilobase (kb) cDNA of MART-1 was cloned into a vector and the resulting plasmid, deposited with the American Type Culture Collection (ATCC) 12301 Parklawn Drive, Rockville, MD 20852 USA on April 14, 1994, and given ATCC Deposit Number 75738. The cloning of MART-1 is disclosed in KawaKami et al J. Exp. Med. 180:347-352, 1994 and Serial No. 08/231,565 filed April 22, 1994. The full length MART-1 nucleic acid sequence can be isolated from the pCRII plasmid by digestion with HINDII and XhoI restriction enzymes. This 1.6kb nucleic acid sequence or

portions thereof can then be incorporated into the genome of the recombinant viruses described herein along with an immunostimulatory gene or genes.

In another embodiment the TAAs are CA-19-A (pancreatic cancer), CA-125 (ovarian cancer), PSA (prostate cancer), erb-2 (breast cancer, CA-171A) and the like (Boon et al. Ann. Rev. Immunol 12:337, 1994).

The present invention is in no way limited to the genes encoding the above listed TAAs. Other TAAs may be identified, isolated and cloned by methods known in the art such as those disclosed in U.S. Patent No. 4,514,506.

Genes encoding an antigen of a disease causing agent in which the agent is a pathogenic microorganism in mammals and include viruses such as HIV (GP-120, p17, GP-160 antigens), influenza (NP, HA antigen), herpes simplex (HSVdD antigen), human papilloma virus, equine encephalitis virus, hepatitis (Hep B Surface Antigen) feline leukemia virus, canine distemper, rabies virus, and the like. Pathogenic bacteria include but are not limited to Chlamydia, Mycobacteria, Legioniella and the like. Pathogenic protozoans include but are not limited to malaria, Babesia, Schistosoma, Toxiplasma, Toxocara canis, and the like. Pathogenic yeast include Aspergillus, invasive Candida, and the like. In a preferred embodiment the pathogenic microorganism is an intracellular organism.

Immunostimulatory Molecules: Costimulation/Accessory Molecules and Cytokines

The gene from costimulation/accessory molecule and/or gene encoding an a cytokine in combination with a gene encoding an antigen from a disease causing agent is incorporated into the genome of a recombinant virus. Examples of costimulation molecules include but are not limited to B7-1, B7-2, ICAM-1, ICAM-2, LFA-1, LFA-3, CD72 and the like. Examples of cytokines encompassed by the present invention include but are not limited to IL-2, IL-

1, IL-3 through IL-9, IL-11, IL-13 through IL-15, G-CSF, M-CSF, GM-CSF, TNFα, IFNα, IFNγ, IL-10, IL-12, Regulated upon activation, normal T expressed and presumably secreted cytokine (RANTES), and the like. Examples of chemokines encompassed by the present invention include but are not limited to CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MBSA, MIP-1α, MIP-1B and the like.

IL-2 construct

The IL-2 gene of the present invention was made made as disclosed by Taniguchi et al (Structure and expression of a cloned cDNA for human interleukin-2 Nature 302:305, 1983).

In one embodiment the entire IL-2 gene as disclosed in Taniguchi et al is incorporated into the TK gene sequence of vaccinia virus.

The promotor sequence for the IL-2 construct of the present invention is made up of the P synthetic late promotor as disclosed in Davidson et al Nucleic Acid Research 18 (No. 14):4285-4286, 1991.

Also encompassed in the present invention is a chimeric gene containing a pox virus promotor region linked to the coding segment of one or more foreign genes encoding an antigen(s) from a disease causing agent and the coding segment of one or more foreign genes encoding an immunostimulatory molecule(s). The chimeric genes then incorporated into the pox virus genome by homologous recombination in cells that have transfected with a plasmid vector containing the chimeric gene and infected with the pox virus.

In a one embodiment the IL-2 construct of the present invention comprises the recombinant vaccinia containing the IL-2 gene and the P synthetic late promotor and an antigen in the TK region from a disease causing agent in the F13L region of the vaccinia virus genome promotor.

B7 Construct

Co-stimulatory molecules of the B7 family (namely

B7.1, B7.2, and possibly B7.3) represent a more recently discovered, but important group of molecules. B7.1 and B7.2 are both member of the Ig gene superfamily. These molecules are present on macrophages, dendritic cells, monocytes, i.e., antigen presenting cells (APCs). If a lymphocyte encounters an antigen alone, with co-stimulation by B7.1, it will respond with either anergy, or apoptosis (programmed cell death); if the co-stimulatory signal is provided it will respond with clonal expansion against the target antigen. No significant amplification of the immune response against a given antigen occurs without co-stimulation (June et al. (Immunology Today 15:321-331, 1994); Chen et al. (Immunology Today 14:483-486); Townsend et al. (Science 259:368-370)). Freeman et al. (J. Immunol. 143:2714-2722, 1989) report cloning and sequencing of B7.1 gene. Azuma et al. (Nature 366:76-79, 1993) report cloning and sequencing B7.2 gene.

In one embodiment the B7.1 gene was inserted into the Hind F13L region of the vaccinia virus, with the β-gal placed in the TK region. The construct for B7.2 and B7.1/B7.2 in conjunction with a tumor antigen are prepared in the same fashion as B7.1.

In another embodiment the B7 gene is inserted into the TK region of vaccinia virus and the gene encoding β-gal inserted in the Hind F13L region of the vaccinia virus.

The IFNγ construct, TNFα construct, GM-CSF construct and ICAM-1 construct were constructed as disclosed in Davidson et al Nucleic Acid Research 18 (No. 14):4285-4286, 1991.

The present invention also encompasses methods of treatment or prevention of a disease caused by the disease causing agents disclosed here.

In the method of treatment, the administration of the recombinant virus of the invention may be for either

"prophylactic" or "therapeutic" purpose. When provided prophylactically, the recombinant virus of the present invention is provided in advance of any symptom. The prophylactic administration of the recombinant virus serves to prevent or ameliorate any subsequent infection or disease. When provided therapeutically, the recombinant virus is provided at (or shortly after) the onset of a symptom of infection or disease. Thus the present invention may be provided either prior to the anticipated exposure to a disease causing agent or after the initiation of the infection or disease.

The genetic definition of tumor-specific antigens allows for the development of targeted antigen-specific vaccines for cancer therapy. Insertion of a tumor antigen gene in the genome of viruses in combination with a immunostimulatory molecule is a powerful system to elicit a specific immune response in terms of prevention in patient with an increased risk of cancer development (preventive immunization), prevention of disease recurrence after primary surgery (anti-metastatic vaccination), or as a tool to expand the number of CTL in vivo, thus improving their effectiveness in eradication of diffuse tumors (treatment of established disease). Finally, recombinant viruses of the present invention can elicit an immune response in patient that is enhanced ex vivo prior to being transferred back to the tumor bearer (adoptive immunotherapy).

The term "unit dose" as it pertains to the inoculum refers to physically discrete units suitable as unitary dosages for mammals, each unit containing a predetermined a quantity of recombinant virus calculated to produce the desired immunogenic effect in association with the required diluent. The specifications for the novel unit dose of an inoculum of this invention are dictated by and are dependent upon the unique characteristics of the recombinant virus and the particular immunologic effect to

be achieved.

The inoculum is typically prepared as a solution in tolerable (acceptable) diluent such as saline, phosphate-buffered saline or other physiologically tolerable diluent and the like to form an aqueous pharmaceutical composition.

The route of inoculation may be intravenous (I.V.), intramuscular (I.M.), subcutaneous (S.C.), intradermal (I.D.) and the like, which results in eliciting a protective response against the disease causing agent. The dose is administered at least once. Subsequent doses may be administered as indicated.

In providing a mammal with the recombinant virus of the present invention, preferably a human, the dosage of administered recombinant virus will vary depending upon such factors as the mammal's age, weight, height, sex, general medical condition, previous medical history, disease progression, tumor burden and the like.

In general, it is desirable to provide the recipient with a dosage of recombinant virus in the range of from about 10^5 to about 10^{10} plaque forming units/mg mammal, although a lower or higher dose may be administered.

The recombinant viral vector can be introduced into a mammal either prior to any evidence of cancers such as melanoma or to mediate regression of the disease in a mammal afflicted with a cancer such as melanoma. Examples of methods for administering the viral vector into mammals include, but are not limited to, exposure of cells to the recombinant virus ex vivo, or injection of the recombinant virus into the affected tissue or intravenous S.C., I.D. or I.M. administration of the virus. Alternatively the recombinant viral vector or combination of recombinant viral vectors may be administered locally by direct injection into the cancerous lesion or topical application in a pharmaceutically acceptable carrier. The quantity of recombinant viral vector, carrying the nucleic acid

sequence of one or more TAAs to be administered is based on the titer of virus particles. A preferred range of the immunogen to be administered is 10^5 to 10^{10} virus particles per mammal, preferably a human.

After immunization the efficacy of the vaccine can be assessed by production of antibodies or immune cells that recognize the antigen, as assessed by specific lytic activity or specific cytokine production or by tumor regression. One skilled in the art would know the conventional methods to assess the aforementioned parameters. If the mammal to be immunized is already afflicted with cancer or metastatic cancer the vaccine can be administered in conjunction with other therapeutic treatments.

In one method of treatment, autologous cytotoxic lymphocytes or tumor infiltrating lymphocytes may be removed from the patient with cancer as disclosed in U.S. Patent No. 5,126,132 and U.S. Patent No. 4,690,915. The lymphocytes are grown in culture and antigen specific lymphocytes expanded by culturing in the presence of the recombinant virus of the present invention. The antigen specific lymphocytes are then reinfused back into the patient.

The present invention also encompasses combination therapy. By combination therapy is meant that the recombinant virus containing one or more genes encoding one or more antigens associated with one or more disease agents and one or more genes encoding one or more immunostimulatory molecules is administered to the patient in combination with other exogenous immunomodulators or immunostimulatory molecules, chemotherapeutic drugs, antibiotics, antifungal drugs, antiviral drugs and the like alone or in combination thereof. In one embodiment the combination therapy includes a recombinant virus and exogenous IL2. In another embodiment the combination therapy includes a recombinant virus encoding one or more

exogenous antigens and exogenous IL-10. Examples of other exogenously added agents include exogenous IL-2, IL-6, IL-12, GM-CSF, interferon, IL-10, tumor necrosis factor, RANTES (Promega, G5661), cyclophosphamide, and cisplatin, gancyclovir, amphotericin B and the like.

This invention further comprises an antibody or antibodies elicited by immunization with the recombinant virus of the present invention. The antibody has specificity for and reacts or binds with the antigen of interest. In this embodiment of the invention the antibodies are monoclonal or polyclonal in origin.

Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules or those portions of an immunoglobulin molecule that contain the antigen binding site, including those portions of immunoglobulin molecules known in the art as F(ab), F(ab'); $F(ab')_2$ and F(v). Polyclonal or monoclonal antibodies may be produced by methods known in the art. (Kohler and Milstein (1975) Nature 256, 495-497; Campbell "Monoclonal Antibody Technology, the Production and Characterization of Rodent and Human Hybridomas" in Burdon et al. (eds.) (1985) "Laboratory Techniques in Biochemistry and Molecular Biology," Volume 13, Elsevier Science Publishers, Amsterdam). The antibodies or antigen binding fragments may also be produced by genetic engineering. The technology for expression of both heavy and light chain genes in E. coli is the subject of the PCT patent applications: publication number WO 901443, WO 901443 and WO 9014424 and in Huse et al. (1989) Science 246:1275-1281.

In one embodiment the antibodies of this invention are used in immunoassays to detect the novel antigen of interest in biological samples.

In one embodiment, the MART-1 antibodies of this invention generated by immunization with recombinant

vaccinia virus expressing MART-1 and B7.1 are used to assess the presence of the MART-1 antigen from a tissue biopsy of a mammal afflicted with melanoma using immunocytochemistry. Such assessment of the delineation of the MART-1 antigen in a diseased tissue can be used to prognose the progression of the disease in a mammal afflicted with the disease or the efficacy of immunotherapy. Conventional methods for immunohistochemistry are described in (Harlow and Lane (eds) (1988) In "Antibodies A Laboratory Manual", Cold Spinning Harbor Press, Cold Spring Harbor, New York; Ausbel et al. (eds) (1987). In Current Protocols In Molecular Biology, John Wiley and Sons (New York, New York).

In another embodiment the antibodies of the present invention are used for immunotherapy. The antibodies of the present invention may be used in adoptive immunotherapy.

In providing a patient with the antibodies or antigen binding fragments to a recipient mammal, preferably a human, the dosage of administered antibodies or antigen binding fragments will vary depending upon such factors as the mammal's age, weight, height, sex, general medical condition, previous medical condition and the like.

In general, it is desirable to provide the recipient with a dosage of antibodies or antigen-binding fragments which is in the range of from about 1 mg/Kg to about 10 mg/Kg body weight of the mammal, although a lower or higher dose may be administered.

The antibodies or antigen-binding fragments of the present invention are intended to be provided to the recipient subject in an amount sufficient to prevent, lessen or attenuate the severity, extent or duration of the disease or infection.

Example 1

Construction and Characterization of Recombinant Vaccinia Viruses Expressing Murine B7 (mB7) and Additional Foreign Proteins

Various methods for inserting foreign genes into a vaccinia virus expression vector are known in the art (Smith, G.L. et al, Vaccinia Virus Expression Vectors: Construction, Properties and Applications Bio Techniques Nov/Dec:306-311, 1984; Flexner, C. et al. Expression of Human Interleukin-1 by live recombinant vaccinia virus. Vaccines 87, Cold Spring, Harbor Lab., pp 380-383; U.S. Patent No. 4,738,846). These as well as other techniques may be employed to construct the recombinant virus of the present invention. Recombinant vaccinia viruses expressing mB7 and additional extrinsic proteins were constructed. The mB7 and additional foreign genes were inserted (by homologous recombination) into the vaccinia VP37 (Hind III F) and TK (Hind III J) genes. For control viruses, the mB7 gene was replaced by the measles HA (mHA) gene.

Construction and Characterization of Vaccinia Recombinant Viruses Expressing mB7-1, mB7-2 and mHA.

The construction of initial recombinants expressing the above genes employed the plaque formation selection system in which the foreign gene is directed into the vaccinia F13L (VP37) loci. Briefly, the foreign gene is cloned into a vaccinia transfer plasmid (pRB21) adjacent to the synthetic early late promoter (E/L) and flanked by DNA homologous to the F13L region in vaccinia. A full-length F13L gene (under control of its authentic promoter) is also situated within the flanking regions. The mB7-1, Mb7-2 and mHA were individually cloned adjacent to E/L within pRB21. A virus that has a plaque deficient phenotype (due to deletion of the F13L plaque forming gene) was used for transfection and the proceeding homologous recombination of plasmid DNA. Transfections

were carried out according to standard published procedures. Recombinant viruses were readily identified according to their plaque positive phenotype, in contrast to the plaque deficient non-recombinants.

Partially plaque purified recombinants from each transfection were consequently characterized using antibodies specific for the individual foreign protein expressed. This was carried out by direct immuno-staining of plaques on unfixed monolayers of BS-C-1 cells. As cells were unfixed this also indicated that the target proteins were expressed on the cell surface. For the majority of studies rat anti-mB7-1 and B7-2 MAbs (Pharmagen) were used to identify surface expression of these proteins. The vaccinia recombinants were plaque purified at least 4 times on BS-C-1 cells (African Green monkey kidney cell line American Type Culture Collection (ATCC Accession No. CCL 26) followed by plaquing on STO cells embryonic mouse fibroblast, Accession No. ATCC/CRL 1503 in the presence of 6-thioguanine (TG) (Sigma Chemical Co.) which selects against unstable single cross-over recombinants. Recombinants were again plaque purified on BS-C-1 cells at least once before stocks were grown in HeLa Spinner cells. The virus was semi-purified by ultracentrifugation through a 36% sucrose cushion. Aliquots were prepared and stored at 80°C. A single aliquot from each batch was thawed, sonicated and titred on BSC-1 cells. Infected monolayers were immunostained to determine continued expression of foreign proteins.

The B7-1 recombinant (v.MCB7.1) was further characterized in a FACS analysis to illustrate surface expression and its ability to bind CTLA4-Ig (B7-1 T-cell receptor) and by Western blot analysis using the anti-B7-1 antibody.

The B7-1 recombinant has been shown to be attenuated compared to wild-type WR, in a mouse LD^{50} assay.

Construction and Characterization of Vaccina Recombinants

mB7-1 , mB7-2 or mHa Co-expressing Additional Genes

Additional genes were inserted into the TK region of the above recombinants. The following is a list of recombinants constructed and characterized (the first part of the code indicates the gene situated in the F13L loci under control of the E/L promoter and latter abbreviation denotes genes in the TK region):

Code	Genes Expressed and Promoters
McB7-1	mB7-1, P. E/L
McB71-1/NP	mB7-1, P. E/L Influenza NP, P7.5K
McB7-1β-gal	mB7.1, P.E/L B-gal. P.7.5K
McB7-1/ova	mB7-1, P.E/L Ovalbumin, P11.K B-gal, 11k
McB7-1/P1A	mB7-1, P.E/L Murine P1A, P7.5K β-gal. P.11k
McB7-1/B7-2	mB7-1, P.E/L B-gal, P.11k
McMHA	Measle Hemaggluttin, P.E./L
McMHA/NP McMHA/β-gal	
McMHA/ova McMHA/P1A	TK inserted, details as above
McMHA/B7-2	mB7-2, 7.5K B-gal. P.11k
McB7-2	B7-2. P.E./L

McB7-2/β-gal	B7-2, P.E./L B-gal. P.11k

A recombinant vaccinia virus containing the genes for B7.1 and the tumor associated antigen, β-gal was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852 USA under Accession No. VR2485 according to terms of the Budapest Treaty. A recombinant vaccinia virus containing the genes for IL-2 and β-gal was deposited with ATCC under Accession No. VR2486.

Sucrose cushion purified stocks of all characterized recombinant viruses have been prepared.

The above method is also employed to construct other recombinant virus using the other previously mentioned viruses such as fowlpox virus in combination with other foreign antigens and immunostimulatory molecules.

Example 2

Recombinant Fowlpox Virus Expressing A TAA, LacZ and B7.1

A recombinant fowlpox virus (rFPV), which is replication incompetent in mammalian cells, is constructed that express the model TAA, lacZ encoding β-gal, and the immunostimulatory molecule, B7.1 under the influence of the 40K vaccinia virus early/late promotor.

The POXVAC-TC (Schering Corp.) strain of FPV is used. FPV is propagated on primary chick embryo dermal cultures (Jenkins, S. et al. AIDS Res. Hum. Retroviruses, 7:991, 1991). Foreign sequences are inserted into FPV by homologous recombination as previously described (Jenkins et al. 1991). Recombinant fowlpox contains the *E. coli* lacZ gene and the B7.1 gene under the control of the vaccinia virus 40K promotor (designed H6 in Rosel, J. et al. J. Virol. 60:436, 1986), inserted into the BAM HI J region of the FPV genome.

Example 3

Recombinant Modified Vaccinia Ankara (MVA) Expressing the TAA lac Z and IC-2

Modified Vaccinia Ankara (MVA) strain is used as the expression vector. MVA was derived from vaccinia virus (WT) by over 570 serial passages in chicken embryo fibroblast cells (CEF) (Mayr, A. et al. Infection 3:6-14, 1975). The resulting MVA strain lost the capacity to productively infect mammalian cells (Altenburger, W. et al. Arch. Virol 105:15-27, 1989; Meyer, H. et al. J. Gen. Virol 72:1031-1038, 1991). The expression of late, as well as early, viral genes is unimpaired in human cells despite the inability of MVA to produce infectious progeny (Sutter and Moss, Proc. Natl. Acad. Sci. USA 89:10847-10851, 1992).

An insertion plasmid is constructed with the lac Z gene and the B7.1 gene under the control of the vaccinia virus late promotor P11 to allow homologous recombination at the site of a naturally occurring 3500-base-pair deletion within the MVA genome. MVA recombinants are isolated and propagated in permissive avian cells as described in Sutter and Moss 1992 and Sutter et al. Virology 1994. The expression of β-gal and B7.1 upon infection of nonpermissive human cells is detected by methods described herein.

Example 4

Effect Of Inoculation Of Various Recombinant Vaccinia Virus Constructs On Primary And Secondary Responses Against Vaccinia Or β-Galactosidase

Materials and Methods

5 BALB/c (H-2^d) mice per group were inoculated i.v. or s.c. with different doses the following vaccinia recombinant (rVV): IL-2 rVV; GM-CSF-rVV; TNFα-rVV; IFNγ-rVV.

In all these constructs the mouse model tumor

associated antigen, β-Gal was under the control of the p7.5-kD (E/L) promoter (Cahran, M.A. et al J. Virol. 54 (No. 1):30-37, 1995) while the cytokine production was driven by $p_{synthetic\ late}$ promoter (Davidson et al Nucleic Acid Research 18 (No. 14):4285-5286, 1991). All these rVV were generated by recombination in the TK region of vaccinia using the plasmid pMJ601 described in Davison et al Nucleic Acids Research 18 (No. 14):4285-5286, 1991.

VJS6

In this construct, containing the E6 protein from Human Papilloma Virus (HPV) a pE/L promoter (the "synthetic superpromoter") is placed just upstream the p7.5 promoter controlling the β-Gal expression and oriented in the same direction.

Control Vaccine

Control vaccinia (crude 19, NP-VV) does not expressing β-Galactosidase.

Primary in vivo Responses and Secondary in vitro Responses

Female BALB/c mice, 8-12 weeks old, were obtained from the Animal Production Colonies, Frederick Cancer Research Facility, NIH, Frederick, MD. Primary lymphocyte populations were generated by injecting BALB/c mice i.v. or subcutaneously with 10^6-10^7 plaque forming units (pfu) of recombinant virus (2 doses in some cases). To assay for primary *in vivo* responses spleens were harvested on day six, dispersed into a single cell suspension and tested for their ability to lyse β-gal expressing and control targets in a six hour ^{51}Cr release assay. Secondary *in vitro* effector populations were generated by harvesting the spleens of mice 21 days after immunization with recombinant virus and culturing single cell suspensions of splencytes in T-75 flasks (Nunc, Denmark) at a density of 5.0 x 10^6 splenocytes/ml. with 1 μg/ml of antigenic peptide in a total volume of 30 ml of culture medium consisting of RPMI 1640 with 10% fetal calf serum (both from Biofluids) that contained 0.1 mM non-essential

amino acids, 1.0mM sodium pyruvate (both from Biofluids) and 5 x 10^{-5} M 2-mercaptoethanol (GIBCO/BRL, Rockville, MD) in the absence of IL-2. Six days later splenocytes were harvested and washed in culture medium before testing in a ^{51}Cr release assay.

^{51}Cr release assay

Six-hour ^{51}Cr release assays were performed as previously described (Restifo, N. et al. J. Exp. Med. 177:265, 1993). Briefly, 1 x 10^6 target cells were incubated with 200mCi $Na^{51}CrO_4$ (^{51}Cr) for ninety minutes. Peptide pulsed targets were incubated with 1 μg/ml (which is roughly 1μM) of antigenic peptide (for β-gal = TPHPARIGL peptide) during labeling as previously described (Restifo, N. et al. J. Immunol 147:1453, 1991). Target cells were then mixed with effector cells for six hours at the effector to target (E:T) ratios indicated. The amount of ^{51}Cr released was determined by γ-counting and the present specific lysis was calculated from triplicate samples as follows: [(experimental cpm - spontaneous cpm)/(maximal cpm - spontaneous cpm)] x 100.

In vivo protection and treatment studies

For *in vivo* protection studies, mice were immunized with recombinant virus 21 days before a subcutaneous challenge with 10^4 tumor cells or an intravenous challenge with 5 x 10^5 tumor cells, as previously described (31). After tumor challenge all mice were randomized. Mice receiving subcutaneous tumor were measured twice a week. When tumors developed, they all grew progressively and were lethal. Mice were euthanized, however, when they were moribund. All mice that appear as long term survivors had no palpable tumor. Mice receiving intravenously administered tumor were euthanized on day 12 and randomized before counting lung metastases in a blinded fashion as previously described (2).

For *in vivo* treatment studies, un-irradiated BALB/c

mice were challenged with either 10^5 or 5 x 10^5 tumor cells intravenously in order to establish pulmonary metastases. Mice were subsequently vaccinated with 10^7 PFU of the designated recombinant virus intravenously or subcutaneously on days three or six. Mice receiving intravenously administered tumor were euthanized on day 12 and randomized before counting lung metastases in a blinded fashion.

The following targets were used in each assay:

CT26 = murine H-2^d adenocarcinoma

CT26 gal = murine H-2^d adenocarcinoma expressing β-Gal. CT26 gal also expresses higher levels of class I MHC molecules.

E22 = murine H-2^d thymoma expressing β-Gal.

CT26 + peptide = CT26 pulsed with TPHPARIGL (876-884) L^d -restricted β-gal peptide

CT26-vac = CT26 infected with crude 19 vaccinia

Lytic Units 30% were calculated for the anti-vaccinia cytotoxic response. Lytic units 30% (L.U. 30) indicate the number of effector cells necessary to give a 30% lysis of 10,000 target cells.

Example 5

Cytokine Secretion After Infection Of BSC-1 Cells With Various Recombinant Vaccinia Virus Constructs

Duplicate wells of 10^5 BSC1 in 1 ml of 2.5% fetal calf serum (FCS) medium RPMI 1640 with antibiotics (24 well plates) were infected with VJS6, rVV-IL-2, rVV-GM-CSF, rVV-IFNγ or rVV-TNFα. At the end of incubation at 27°C for 24 hours, supernatants were removed, centrifuged, and the concentration of cytokine determined as shown in Table 1. Values are expressed in pg/ml.

The concentration of each cytokine was determined using commercially available detection kits for GM-CSF, IFNγ; IL-2 and TNFα.

The results show that high concentrations of each cytokine were detected. The highest concentration of cytokine produced was IL-2 from BSC-1 cells infected with rVV-IL2 (Table 1).

Table 1

DETECTION OF CYTOKINES SECRETED AFTER INFECTION OF BSC1 CELLS WITH DIFFERENT rVV

	MOI 12h				MOI 36h			
rVV	1:1	0.1:1	0.01:1	TITER[a]	1:1	0.1:1	0.01:1	TITER[a]
				GM-CSF detection kit				
VJS6	<15.6	<15.6	<15.6	-	<15.6	<15.6	<15.6	-
IL-2	<15.6	<15.6	22	-	<15.6	<15.6	<15.6	-
GMCSF	>250	>250	>250	$5.1X10^5$	>250	>250	>250	$4.2x10^6$
IFNγ	<15.6	<15.6	<15.6	-	<15.6	<15.6	<15.6	-
TNFα	<15.6	<15.6	<15.6	-		<15.6	<15.6	-
				IFNγ detection kit				
VJS6	<47	<47	<47	-	<47	<47	<47	-
IL-2	<47	<47	<47	-	<47	<47	<47	-
GMCSF	<47	<47	<47	-	<47	<47	<47	-
IFNγ	>12000	9000	1000	56000	>12000	>12000	>12000	>67000
TNFα	<47	<47	<47	-	<47	<47	<47	-
				IL-2 detection kit				
VJS6	<34	<34	<34	-	<34	<34	<34	-
IL-2	>850	>850	>850	$2x10^6$	>850	>850	>850	$3.9x10^6$
GMCSF	<34	<34	50	-	54	63	110	-
IFNγ	<34	121	135	-	94	97	132	-
TNFα	<34	<34	<34	-	<34	<34	<34	-
				TNFα detection kit				
VJS6	<30.2	<30.2	<30.2	-	<30.2	<30.2	<30.2	-
IL-2	<30.2	<30.2	<30.2	-	<30.2	<30.2	<30.2	-
GMCSF	<30.2	<30.2	<30.2	-	<30.2	<30.2	<30.2	-
IFNγ	<30.2	<30.2	<30.2	-	<30.2	<30.2	<30.2	-
TNFα	2050	310	295	2050	>2450	>2450	>2450	135000

a. Supernatants from 1:1 MOI (multiplicity of infectivity) infected cells, that were expected to exceed the highest point of the standard curve, were serially diluted (1:10 dilution) to calculate the precise cytokine concentration.

b. Only one of the two wells was positive.

Example 6

β-Galactosidase Production After BSC1 Cell Infection With Various Vaccinia Virus Constructs

One X10^5 BSC1 cells were infected with 1:1 MOI of either rVV-VJS6, rVV-IL2, rVV-GM-CSF- rVV-TNFα or rVV-IFNγ. At this MOI the cytopathic effect was less pronounced at time end of incubation, and presumably no leakage of the enzyme had occurred. Pelleted cells were subject to three freeze-thaw rounds to release the cytoplasm content, cellular debris was removed and the supernatant used for detection of galactosidase activity on the substrate O-nitrophenyl-β-D-galactopyranoside (ONPG) using a Promega Kit No. E2000 (Promega, Madison, Wisconsin). One unit of galactosidase hydrolyze 1μM ONPG per minute at pH 7.5 at 37°C. Duplicate wells were run on each sample. The results from two different experiments are shown in Figure 1. The control vaccinia, NP-VV, did not express β-gal. VJS6 samples were serially diluted to fit within the depicted range. The results show a 1 log difference in the β-Gal enzymatic activity of VJS6 compared to the other recombinant vaccinia virus. The results as shown in Figure 1 parallel with the kinetics of blue staining with X-gal in plaque assays **(data not shown)**.

Example 7

Primary Response Of Mice At Day 6 After Injection I.V. With Various Vaccinia Virus Constructs

5 BALB/c mice were injected intravenously (I.V.) with 5x10^6 plaque forming units (pfu) of the following recombinant vaccinia virus: VJS6, IL2-rVV, GM-CSF-rVV, TNFα-rVV OR IFNγ-rVV. At day 6, the spleens were removed and tested against the following target cells as described in Example 4: CT26, CT26 peptide, CT26 gal, E22, and CT26 vac.

Figure 2 shows the peak primary CTL response. IL-2 and GM-CSF produced by rVV during infection notably

enhanced the primary CTL response against vaccinia determinants. The spleen cells also gave a weak primary response toward β-gal.

Example 8

Secondary Cultures From Mice Infected I.V. With Various Recombinant Vaccinia Virus Constructs

Mice were infected with various recombinant vaccinia virus constructs as described in Example 4. Splenocytes were harvested at day 6 or day 14 after inoculation with the rVV. Secondary cultures were generated by 6 days incubation of $6x10^6$ splenocytes/ml in complete medium containing 1 μg/ml TPHPARIGC peptide as indicated in Example 4. The results showed that CTL from VJS6, IL2-rVV and GM-CSF-rVV vaccinated mice could not be restimulated with an β-gal peptide 6 days after viral injection (Figure 3). The TNF-rVV response of CTLs was characterized by the induction of a long-lasting non-specific cytotoxicity (Figures 3 and 4). The GM-CSF production had a negative effect on the anti-β-gal immune response of CTLs.

Example 9

Primary Response Of Mice At Day 6 After Injection S.C. With Various Recombinant Vaccinia Virus Constructs

5 BALB/c mice were injected subcutaneously (S.C.) with $5x10^6$ pfu of the following recombinant vaccinia virus: VJS6, IL2-rVV, GM-CSF-rVV, TNFα-rVV or IFNγ-rVV. At day 6, the spleens were removed and tested against the following target cell as described in Example 4: CT26, CT26 peptide, CT26 gal, E22 and CT26 vac.

Figure 5 shows in all cases, each cytokine helped to increase the response to vaccinia in comparison to VJS6 using the CT26 target, with the IL-2-rVV treatment being the highest. No lysis was obtained with the other target cells. This indicates that the kinetics of the immune response induced by S.C. inoculation may be different or delayed in comparison to I.V. inoculation. However,

higher doses and/or different time intervals is expected to increase the magnitude of the primary response against vaccinia using the subcutaneous route of inoculation.

The lytic units (L.U.) 30% was determined by the number of effector cells necessary to give 30% lysis of 10,000 target cells using the CT26-vac cells as targets, Figure 6 shows the results of this titration. The L.U./spleen ($x10^3$) of each rVV is as follows: Gm-CSF= 124.3; VJS6 - 14.9; IFN-γ - 5.06; TNFα = 2.88 and IL-2 = 96.2.

Example 10

Secondary Cultures From Mice Infected S.C. With Various Recombinant Vaccinia Virus Constructs

Mice were infected with various recombinant vaccinia virus constructs as described in Example 4. Splenocytes were harvested at day 14 after inoculation with rVV. Secondary cultures were generated by 6 days incubation of $6x10^6$ splenocytes/ml in complete medium containing 1 μg/ml TPHPARIGC peptide as indicated in Example 4. The results as depicted in Figure 7 show a high level of non-specific response to each target cell regardless of the effector cell.

Example 11

Treatment Of Established Pulmonary Metastases With Recombinant Vaccinia Viruses Secreting Different Cytokines

The three-day pulmonary metastases mouse model was used to evaluate the efficacy of treatment using rVV. Nontreated mice normally succumb to the metastases in 11-14 days using this model system.

Mice were injected I.V. on day 0 with $5x10^5$ tumor cells (CT26 or CT26 β gal). Three days later they received an I.V. injection of $5x10^5$ PFU of VJS6, IL-2-rVV, GM-CSF-rVV, IFNα-rVV, TNFγ-rVV or Hanks' Balanced Salt Solution (HBSS) (control mice). The mice were randomized, and the lungs were harvested after 12 days. The number of

metastases was determined by counting in a blinded fashion.

Table 2 shows that moderate doses of IL2 produced by the rVV was effective in treatment of 3 day old metastases induced by CT26 β gal.

Table 2

DAY 3 TREATMENT OF ESTABLISHED PULMONARY METASTASES WITH RECOMBINANT VACCINIA VIRUSES SECRETING DIFFERENT CYTOKINES

	CT26		CT26 βgal	
rVV treatment	average # metastases	metastases/ mouse	average # metastases	metastases/ mouse
none*	>500	>500 x 5	>500	>500 x 5
VJS6	>500	>500 x 5	186	162, 116, 115, 362, 175
IL-2 rVV	452.4	500 x 4, 262	11.4	13, 2, 24, 12, 7
GM-CSF rVV	470.2	500 x 4, 351	373.6	500 x 2, 405, 283, 179
IFNγ rVV	>500	>500 x 5	232.8	280, 97, 190, 260, 337
TNFα rVV	>500	>500 x 5	361.2	>500 x 2, 389, 258, 159

* control mice were injected with HBSS alone

Example 12

Treatment Of Established Pulmonary Metastases With Recombinant Vaccinia Virus Secreting Different Cytokines Plus Exogenous IL2

Mice were injected with tumor cells and rVV as indicated in Example 4. Treatment with exogenous rIL-2 (15,000 cetus units, twice a day, I.P.) was started 6 h after rVV injection and protracted for 5 days. Lungs were harvested after 12 days.

Moderate doses of IL2 (produced by rVV or exogenously administered together with rVV) were effective in treatment of 3 day old metastases induced by CT26 β- gal. (Table 3)

Table 3

DAY 3 TREATMENT OF ESTABLISHED PULMONARY METASTASES WITH RECOMBINANT VACCINIA VIRUSES PRODUCING DIFFERENT CYTOKINES PLUS EXOGENOUS IL-2

	CT26		CT26 βgal	
rVV treatment	average # metastases	metastases/ mouse	average # metastases	metastases/ mouse
none[a]	>500	>500 x 5	>500[b]	>500 x 5
VJS6	>500	>500 x 5	405.2	>500 x 3, 267, 259
IL-2 rVV	437.4	500 x 3, 267, 420	127.4[c]	184, 7, 126, 91, 229
rIL-2	>500	>500 x 5	>500	>500 x 5
rIL-2 + VJS6	406.4	>500 x 3, 298, 234	20.8[c]	43, 6, 0, 52, 3
GM-CSF rVV	413.2	>500 x 3, 163, 403	500	500 x 5
IFNγ rVV	447.6	>500 x 4, 238	412.2	500 x 4, 66

[a] control mice were injected with HBSS alone
[b] all mice in this group died between days 11 and 12 (before lung harvest)
[c] p value between the data in the frame is 0.07

Example 13

Active Immunotherapy Using Exogenous IL-2 Together With rVV Expressing A Tumor Associated Antigen

A recombinant vaccinia virus expressing a Tumor Associated Antigen (TAA) was constructed as described in Example 1.

Mice were injected with 5 x 10^5 of CT-26 expressing β-gal via an I.V. route of administration. Three days later the mice were injected with rVV-TAA-β-gal, alone or in combination with exogenous IL2. [High dose (HD) exogenous IL-2 (100,000 I.U., I.P., BID x 3d) or low dose (IL) IL-2 (15,000 I.U., I.P., BID x 3d)] was administered as indicated and then randomized.

The number of mice surviving and survival time in days was monitored. Those mice receiving rVV-TAA plus exogenous IL2 survived. (Figure 8)

Example 14

Figure 9 shows the survival of mice with non-β-gal expressing tumor established after immunotherapy with different recombinant vaccinia vectors. Mice were injected with 5 x 10^5 CT 26 tumor cells (non-β-gal) via I.V. route of administration. On day three they were injected with 10^3 pfu of the following rVV which also contained the β-gal gene: IL-2rVV, TNFα-rVV, VJS6, GM-CSFrVV. The number of mice surviving and survival time in days was monitored. No difference in survival was observed in any of the treatment groups.

Figure 10 shows survival in mice with an established β-gal expressing tumor, CT26.25 after immunotherapy with different recombinant vaccinia vectors. Mice were injected with 5 x 10^5 CT26.25 (β-gal expressing) via I.V. route of administration. On day three they were injected with 10^3 pfu of the rVV described above. The results show that a clear survival advantage was conferred on those mice treated with the IL-2rVV (also containing the β-gal gene) compared to treatment with any other rVV.

Example 15

Active Treatment Of Established Pulmonary Metastases Using B7 - rVV Construct

All of the following recombinant Vaccinia vectors were constructed by placing both the cytokine gene and the TAA gene in the TK region of the vaccinia vector genome. Promotors varied based on the plasmid used for recombination. The majority of the constructs used the P7.5K promotor.

The three-day pulmonary metastases model was used as described in Example 4. Five x 10^5 tumor cells were injected into mice. Three days later 10^6-10^7 pfu of each vector was injected I.V. Lungs were harvested on day 12.

The results as depicted in Figure 11 and 12 showed that the rVV vectors encoding ß-gal and the

immunostimulatory molecule, B7.1, IL-2 or ICAM-1 profoundly inhibited pulmonary metastases in an antigen specific manner.

The primary response of the mice on Day 9 after injection with tumor cells was determined. The results are depicted in Table 4. The percent lysis of ^{51}Cr labeled target tumor cells is recorded. A significant primary immune response was seen with multiple recombinant vaccinia vectors, most notably B7.1rVV, which correlates with the in vivo treatment response.

Table 4
Primary Response of Mice 6 Days After Injection I.V. With Various Vaccinia Virus Constructs

		Tumor cells			
rVV (pfu) Construct	Effector Target	CT26	CT26/P	C25	CT26/V69
B7.1	100:1	-3.86*	82.99	81.45	21.69
	33:1	-4.86	91.86	69.01	14.49
	11:1	-6.10	61.64	71.75	7.33
	37:1	-8.61	53.99	54.22	2.19
	1.2:1	-9.53	39.93	40.87	3.25
	04:1	-16.57	27.37	26.74	-0.15
B7.1 10^6	100:1	2.75	68.18	5270	70.75
	33:1	-2.05	84.93	66.76	82.89
	11:1	-2.85	41.27	73.12	56.05
	37:1	-3.76	41.93	350.06	42.19
	12:1	-8.05	26.24	31.70	44.97
	0.4:1	-8.52	20.50	35.49	37.13
NA 10^7**	100:1	4.04	79.02	69.65	14.06
	33:1	1.70	72.66	82.36	10.65
	37:1	-1.01	51.75	63.41	7.53
	11:1	-2.75	35.65	42.33	0.37
	12:1	-4.88	34.98	28.49	-11.67
	0.4:1	-6.57	6.14	20.02	-13.66
NA 10^6	100:1	27.40	88.40	121.19	31.48
	33:1	13.47	74.16	112.42	1.41
	11:1	0.53	41.16	97.33	-4.07
	3.7:1	-0.35	8.95	86.27	1.58
	1.2:1	-0.43	-2.05	69.52	371
	04:1	-4.06	-2.59	40.13	-4.37

	MVA 10^8	100:1	12.76	27.43	65.08	12.10
		33:1	8.52	17.62	61.83	8.37
		11:1	3.45	2.95	49.38	4.34
		37:1	0.06	5.76	28.12	3.13
		1.2:1	-1.30	2.43	28.05	-3.60
		0.4:1	-4.26	-0.61	16.78	-7.65
	MVA 10^7	100:1	17.54	71.07	46.31	0.14
		33:1	13.81	70.97	37.10	1.10
		11:1	6.36	44.59	35.00	-0.30
		3.7:1	-2.29	17.29	28.58	-6.94
		1.2:1	-3.54	2.82	27.91	-0.76
		0.4:1	-17.53	-6.63	19.66	-9.87
	Kb 10^7	100:1	24.83	20.58	33.37	34.47
		33:1	20.74	15.35	19.42	34.75
		11:1	12.61	-10.25	9.73	27.77
		37:1	9.82	-11.37	8.51	17.51
		1.2:1	-5.98	-18.23	8.58	17.01
		0.4:1	-10.88	-29.04	5.55	9.10
	Kb 10^6	100:1	11.60	28.14	42.96	-0.32
		33:1	-2.37	16.79	21.60	-1.60
		11:1	-7.83	16.12	-1.67	-7.08
		37:1	-12.70	8.51	-3.28	-11.12
		1.2:1	-18.17	6.15	-11.68	-9.36
		0.4:1	-24.72	-4.83	-17.02	-14.28
	Ld 10^7	100:1	-9.69	86.06	92.89	-5.1
		33:1	-32.65	33.05	53.22	-8.54
		11:1	-39.51	35.26	46.63	-19.29
		37:1	-42.44	15.50	24.28	-17.91
		1.2:1	-47.50	2.17	12.71	-17.45
		0.4:1	-51.77	-1.65	9.44	-30.42
	Ld 10^6	100:1	28.10	56.29	30.10	16.62
		33:1	16.09	41.69	26.49	-5.75
		11:1	-1.22	24.49	24.50	-17.06
		37:1	-3.46	24.04	18.81	-21.05
		1.2:1	-9.65	8.65	13.16	-20.82
		0.4:1	-17.10	2.42	8.34	-27.53
	VJS6 10^7	100:1	29.38	33.59	73.98	22.03
		33:1	24.69	31.48	72.14	24.43
		11:1	20.17	27.87	61.82	13.66
		37:1	16.83	23.47	57.46	7.24

	1.2:1	8.44	17.93	51.60	4.11
	0.4:1	4.01	19.12	33.13	-5.46
VJS6 10^6	100:1	1.53	63.88	63.8	45.57
	33:1	-23.69	35.15	36.13	39.89
	11:1	-27.32	29.65	27.61	29.29
	37:1	-41.54	21.54	21.57	24.26
	1.2:1	-45.02	18.21	16.21	21.06
	0.4:1	-52.85	14.18	8.78	18.50
WB_2m 10^7	100:1	3.50	11.12	23.52	4.69
	33:1	0.32	11.95	10.52	3.46
	11:1	-10.09	5.10	7.51	-2.53
	37:1	-21.06	0.22	1.10	-2.51
	1.2:1	-28.28	-3.99	-3.04	-4.26
	0.4:1	-30.42	-7.84	-11.03	-7.36
WB_2m 10^6	100:1	25.09	16.18	22.86	1.54
	33:1	25.61	6.04	19.48	-2.55
	11:1	22.77	-1.80	14.43	-11.26
	37:1	9.18	-8.25	11.40	-19.36
	1.2:1	3.90	-19.60	10.69	-24.29
	0.4:1	-13.93	-28.67	-1.28	-29.35
HBSS	100:1	6.00	17.33	6.73	-4.50
	33:1	0.30	14.46	6.58	-9.50
	11:1	-10.32	9.65	-9.60	-5.68
	37:1	-14.34	1.66	-17.73	-13.69
	1.2:1	-23.70	-9.03	-30.78	-17.72
	0.4:1	-29.28	-12.56	-38.08	-17.75

* % lysis of ^{51}Cr labeled tumor cells
** NA = neuraminidase recombinant viral vector (rVV), MVA = Ankara non-replicating vaccinia virus rVV, Kb = murine H-$2K^b$ MHC Class I molecule rVV, Ld = murine H-$2L^d$ MHC Class I molecule rVV, WB_2m = murine Beta 2-microglobulin rVV, HBSS = Hank's Balanced Salt Solution

The secondary response of the mice is depicted in Table 5. The results indicate a secondary immune response seen by both VJS6 and B7.1 against the relevant target.

Table 5
Secondary Response of Mice 6 days After
I.V. Injection with Various Vaccinia Virus Constructs

	Tumor cells				
rVV (pfu) Construct	Effector Target	CT26	CT26/P	C25	CT26/V69
HBSS	100:1	30.26*	45.93	37.05	29.95
	33:1	15.83	13.38	10.05	18.24
	11:1	2.59	5.88	4.37	11.39
	37:1	0.83	5.64	-1.68	-1.00
	1.2:1	0.01	2.14	-2.71	4.12
	0.4:1	1.66	6.71	8.61	-0.11
VJS6 10^7	100:1	19.40	72.31	78.94	28.42
	33:1	21.52	83.69	63.82	18.04
	11:1	10.84	62.35	61.87	16.80
	37:1	7.91	44.31	52.99	4.75
	1.2:1	10.24	21.03	18.30	5.72
	0.1:1	4.59	14.14	11.92	10.07
VJS6 10^6	100:1	18.55	48.89	38.21	15.27
	33:1	11.33	41.52	34.52	8.25
	11:1	8.06	26.63	33.00	1.17
	37:1	2.57	16.54	29.26	-2.23
	1.2:1	7.86	14.96	13.57	0.56
	0.4:1	2.05	3.65	9.47	-8.17
Ld 10^{7}**	100:1	0.11	30.56	25.05	-4.91
	33:1	-2.78	25.03	26.24	-0.48
	11:1	2.49	16.01	11.97	-3.99
	37:1	-1.02	7.17	7.28	3.50
	1.2:1	0.52	9.57	4.36	4.06
	0.4:1	3.10	6.23	2.35	3.29
Ld 10^6	100:1	18.65	25.02	14.24	11.70
	33:1	0.63	11.23	7.51	11.36
	11:1	-0.35	7.47	10.24	2.76
	37:1	-0.47	1.38	-0.00	3.32
	1.2:1	-3.19	6.35	5.89	6.16
	0.4:1	4.08	5.70	2.86	4.04
B7.1 10^7	100:1	-10.62	83.64	69.56	-3.17
	33:1	0.20	63.61	59.63	0.23
	11:1	-6.10	40.58	61.77	3.02
	37:1	-1.91	22.29	22.86	-2.5
	1.2:1	-1.38	8.0	10.25	0.22

	0.4:1	4.73	8.39	9.49	2.22
B7.1 10^6	100:1	30.59	59.22	61.29	32.13
	33:1	31.24	74.4	53.99	23.29
	11:1	13.92	41.68	49.63	22.94
	37:1	10.66	31.02	34.86	5.63
	1.2:1	4.87	33.61	6.45	3.73
	0.4:1	8.78	7.66	3.98	7.16
MVA	100:1	28.16	18.13	25.09	30.30
	33:1	33.76	28.62	27.99	20.10
	11:1	28.69	12.63	9.75	9.13
	37:1	6.55	-0.43	12.79	2.78
	1.2:1	8.08	4.47	1.63	-0.45
	0.4:1	5.68	-1.30	1.21	4.60
MVA 10^7	100:1	22.77	32.85	28.05	27.07
	33:1	10.46	28.94	30.49	8.80
	11:1	5.48	24.20	22.32	-3.73
	37:1	0.49	11.9	12.82	4.36
	1.2:1	-0.95	5.29	13.32	6.36
	0.4:1	4.75	-0.73	14.69	9.59

* % lysis of ^{51}Cr labeled tumor cells
** NA = neuraminidase recombinant viral vecot (rVV), MVA = Ankara non-replicating vaccinia virus rVV, Kb = murine H-$2K^6$ MHC Class I molecule rVV, Ld = murine H-$2L^d$ MHC Class I molecule rVV, WB_2m = murine Beta 2-microglobulin rVV, HBSS = Hank's Balanced Salt Solution

Example 16

Clinical Protocol For Phase I/II Trials In Patients With Metastatic Melanoma and Metastatic Breast Cancer of Immunization with a Recombinant Vaccinia Virus Expressing the MAGE-1 Peptide and B7.1

In this protocol patients with advanced melanoma and advanced breast cancer are immunized against MAGE-1, an immunodominant peptide from a cancer antigen in combination with costimulatory/accessory molecule B7-1.

Patient Eligibility

Among other criteria, patients must have evidence of measurable or evaluable metastatic melanoma or breast cancer that has failed standard effective therapy. Patients must have tumors that express the MAGE-1 antigen

as evidenced by PCR or Northern Blot analysis of tumor cell RNA.

Treatment

Melanoma and breast cancer patients are divided into two categories each, those who have previously been vaccinated against smallpox and those who have not. Three patients in each category receive intradermal immunization with 10^8 plaque forming units/ml every four weeks for a total of three doses. Patients are evaluated for toxicity. When three patients in each category have been followed for at least two weeks after the first immunization without achieving grade 3 or 4 toxicity not easily reversible by standard measures then the dose in that plaque category will be escalated to 10^9 plaque forming units/ml intradermally every four weeks for a total of three doses. Fifteen patients in each category will be treated at this dose and carefully evaluation of toxicity, immunologic effects and therapeutic efficacy will be evaluated.

If any patient receiving the 10^8 pfu/ml dose achieves grade 3 or 4 toxicity not easily reversible by standard measures, then an additional three patients will be treated at that dose. If a second patient develops grade 3 or 4 toxicity, not easily managed by standard procedures, then the dose will not be escalated in that category.

Vaccine preparation. The recombinant vaccinia virus used in this protocol is the Wyeth vaccinia virus derived from the New York City Board of Health strain. This is an attenuated virus with an extensive history of previous use in humans. The recombinant virus has the gene coding for the MAGE-1 nine amino acid minimal determinant plus the B7.1 sequence inserted into the viral thymidine kinase gene.

The recombinant virus is produced in a Food and Drug Administration approved facility for the manufacture of GMP grade clinical material.

Vaccination procedure. The recombinant vaccinia virus will be supplied as a sterile, frozen suspension and will be approximately diluted to either 10^8 or 10^9 plaque forming units/ml with phosphate buffered saline.

Vaccination will be performed in the deltoid muscle area. The method of vaccination is summarized as follows:

a) Open the bifurcated needle by catching the butt-end of the needle and gently pulling the point end free.

b) Dip the bifurcated point of the needle into vaccine. The needle will pick up a drop of vaccine in the space between the two points.

c) Using the same bifurcated needle, use the multiple pressure technique by pressing the needle through the vaccine drop on the subject's skin. Fifteen needle pressures will be administered.

d) Alternative upper extremities will be used for subsequent vaccinations.

Patients will be observed for 24 hours after immunization. Body temperature will be measured at 12 hours and at 24 hours and any adverse reactions noted.

Post vaccination evaluation

On day 14 after vaccination, patients will be seen and the following tests obtained:

a) Complete history and physical examination, including any symptoms and progress of reaction from the vaccination.

b) Photograph of the vaccination site.

c) Complete blood count with differential count.

d) Platelet counts.

e) Acute care, hepatic, mineral and thyroid blood chemistry panel.

f) Serum and lymphocytes stored as performed

pretreatment.

Patients will be evaluated at 14 days following the second vaccination and similar procedures will be performed prior to and following the third and final vaccination on day 56.

Patients will have a complete restaging of all sites of disease with appropriate physical examination and x-rays and nuclear medicine studies at the time of one and two months following the final vaccination.

Immunologic studies

Immunologic assessment will be made of the patient's response to vaccinia antigens as well as to the MAGE-1 antigen.

a) All serum samples will be tested for anti-vaccinia antibody by ELISA.

b) All cryopreserved lymphocytes will be tested for response to MAGE-1 antigen using limiting dilution analysis of precursor CTL frequency using the method of Coulie, P et al International Journal of Cancer 50:289-297, 1992.

c) Patients with easily accessible disease may have biopsy under local anesthesia of accessible tumor to study the histopathologic nature of the tumor as well as the isolation of tumor infiltrating lymphocytes for in vitro growth. Tumor infiltrating lymphocytes will be tested for specific reactivity and specific cytokine release against MAGE associated antigens.

Assessment of response

A complete response is defined as the disappearance of all clinical evidence of disease that lasts at least four weeks.

A partial response is a 50% or greater decrease in the sum of the products of the perpendicular diameter of all measurable lesions for at least four weeks with no appearance of new lesions or increase in any lesions.

Minor responses are defined as 25-49% decrease in the sum of the products of the perpendicular diameters of all measurable lesions with no appearance of new lesions and no increase in any lesions.

Any patient with less than a partial response will be considered a non-responder.

The appearance of new lesions or greater than 25% increase in the product of perpendicular diameters of prior lesions following a partial or complete response will be considered as a relapse.

Similar protocols will be followed for the evaluation of other recombinant virus vaccines against cancer. Vaccines to be tested include but are not limited to Recombinant Vaccinia virus encoding MART-1 and IL-2, Recombinant Vaccinia virus encoding MART-1 and B7.1, Recombinant Vaccinia virus encoding GP100 and IL-2, Recombinant vaccinia virus encoding GP100 and B7.1, Recombinant fowlpox virus encoding MART-1 and IL2, recombinant fowlpox virus encoding MART and B7.1 and the like with the appropriate modifications depending on the antigen and virus used.

Example 17

Use of Lymphocytes Sensitized To Immunogenic Peptides Derived From Melanoma Antigens For Therapeutically Treating Mammals Afflicted With Melanoma

T-lymphocytes presensitized to the melanoma antigen may be effective in therapeutically treating mammals afflicted with melanoma. T-lymphocytes are isolated from peripheral blood or melanoma tumor suspensions and cultured in vitro (Kawakami, Y. et al. (1988) J. Exp. Med. 168:2183-2191). The T-lymphocytes are exposed to cells infected with the recombinant virus expressing a melanoma associated antigen and IL2 and/or B7.1 for a period of about to 1-16 hours at a concentration of 1 to 10mg/ml. T-lymphocytes exposed to the antigen will be administered

to the mammal, preferably a human at about 10^9-10^{12} lymphocytes per mammal. The lymphocytes may be administered either intravenously, intraperitoneally or intralesionally. This treatment may be administered concurrently with other therapeutic treatments such as cytokines, radiotherapy, surgical excision of melanoma lesions and chemotherapeutic drugs, adoptive T lymphocyte therapy.

Example 18

Active Immunotherapy with Recombinant Vaccinia Virus Co-expressing B7-1, B7-2 or Both and a Model Tumor Antigen Mediates Regression of Established Pulmonary Metastases

Materials and Methods

Animals

Female BALB/c (H-2^d) mice were obtained from Frederick Cancer Research Center (Frederick, MD). All mice were used at 6 - 8 weeks of age.

Preparation of Cell Lines

CT26 is an N-nitroso-N-methylurethrane induced BALB/c (H-2^d) undifferentiated colon carcinoma as supplied by D. Pardoll (Baltimore, MD). CT26 was cloned to produce a wild type parental tumor line, CT26.WT. The gene for *lac Z* was stably transfected into CT26.WT as previously described herein and in Wang, M. et al J. Immunol. 154(9):4685-4692, 1995. Briefly, a plasmid donated by A.D. Miller containing the gene for β-galactosidase and a neomycin resistance marker were used to construct the LZSN amphotropic retrovirus which was used to transduce CT26.WT. Transductants were selected in G418 media, and subcloned by limiting dilution analysis at 0.3 cells/well. Subclones that expressed β-galactosidase were screened by X-gal staining and in ^{51}Cr release assays with anti-β-galactosidase effectors. The subclone CT26.CL25 was selected for use in all studies because of its stable expression of both β-galactosidase and the class I

molecule H-2 L^d. BS-C-1 or HeLa S^3 cells (ATCC, American Tissue Typing Collection, Rockville, MD CCL 16) were used to expand and titer all viruses. Cell lines were maintained in RPMI 1640, 10% heat inactivated FCS (Biofluids, Rockville, MD), 0.03% L-glutamine, 100 μg/ml streptomycin, 100 μg/ml penicillin and 50 μg/ml gentamicin sulfate (NIH Media Center). CT26.CL25 was maintained in media containing 400 or 800 μg/ml G418 (GIBCO, Grand Island, N.Y.).

Construction and Characterization of Recombinant Vaccinia Viruses

Construction and characterization of recombinant vaccinia viruses containing murine B7-1(v.MCB7-1), B7-2 (v. MCB7-2), B7-1 and B7-2 (v.B7-1/B7-2) and measles hemagglutinin (HA) (v.MCMHA) is as follows. Briefly, the B7-1 gene (a gift from Dr. R. Germain, NIH) and the measles HA gene (a gift from Dr. S. Rozenblatt, Tel Aviv University, Israel) were cloned into the transfer plasmid pRB12 such that they were under control of the VV synthetic early/late promoter. The gene of interest was inserted into the Hind III F region of VV. Methods used for the production and selection of rVV in which the VV thymidine kinase gene was utilized as an insertion site, were similar to those described previously by Earl, P.L. et al. In: Current Protocols in Molecular Biol. Ausubil et al (eds) Greene Publishing Assoc. and Wiley Interscience, 1991, 16.18.1-16.18.10 unless otherwise stated. The v.MCB7-1/β-gal and v.MCMHA/β-gal were constructed by homologous recombination of the plasmid pSC65Δ (a modification of pSC65 in which the E. coli *lac Z* gene is under transcriptional control of the early function of the VV p 7.5k promoter and flanked by VV derived TK DNA) with v.MCB7-1 and v.MCMHA respectively. The v. MCB7-2/β-gal was constructed with both the B7-2 gene and the E. coli *lac Z* gene under transcriptional

control of the early function of the VV p 7.5k promoter and flanked by VV derived TK DNA. The v. MCB7-1/B7-2/β-gal was constructed by homologous recombination of the plasmid pSC65Δ (modified as stated previously to create the v. MCB7-2/β-gal construct) with the v. MCB7-1.

Table 6. Construction of recombinant vaccinia viruses.

Recombinant Vaccinia Virus Code	VP37		Thymidine Kinase	
	Promoter	Gene	Promoter	Gene
v.MCB7-1/β-gal	S.E/L	B7-1	7.5e	β-gal
v.MCMHA/β-gal	S.E/L	MHA	7.5.e	β-gal
v.MCB7-1/NP	S.E/L	B7-1	7.5e	NP
v. MCB7-2/β-gal			7.5e	B7-2, β-gal
v. MCB7-1/B7-2/β-gal	S.E/L	B7-2	7.5e	B7-2, β-gal
v.MCMHA/B7-2/B-gal	S.E/L	MHA	7.5e	B7-2, β-gal

MHA= measle hemagglutinin gene, B7-1 = murine B7-1, B7-2 = murine B7-2, NP = influenza nucleoprotein, β-gal= E.coli β-galactosidase gene, S.E/L = vaccinia synthetic early late promoter, 7.5e = early only function of the vaccinia 7.5k promoter.

Recombinant viruses were simultaneously selected for their TK negative phenotype and ability to express β-galactosidase. Transfer plasmid pGS69 (Smith, G.L. et al Virology 160:336-345, 1987 containing the influenza nucleoprotein (NP) was recombined with v.MCB7-1 to yield v. MCB7-1/NP. This recombinant virus was selected on the basis of a TK negative phenotype. Plaques were then analyzed for expression of NP by immunostaining (Sutter, G. et al Vaccine 12:1032-1040, 1994) and subsequently plaque purified under agar overlay four times before sucrose cushion purified viral stocks were prepared (Earl, P.L. et al Current Protocols in Mol. Biol., ibid (Table

6.).

Preparation of rVV expressing the influenza A/PR/8/34 nucleoprotein (NP) was previously described (Smith, G.L. et al Virology 160:336-345, 1987). In the control construct, v. JS6, the E.coli *lac Z* gene was under the control of the early VV p 7.5k promoter from plasmid pSC65. Murine IL-2 cDNA was amplified by polymerase chain reaction from pBMGNeomIL-2 and ligated into the *Sma I-BamHI* site of a vaccinia expression vector, pMJ601, which contains the b-galactosidase gene under the control of VV early p 7.5k promoter. The other cytokines (GM-CSF, IFN-g, and TNF-a) were inserted into the wild type VV genome with similar procedure. The techniques used in preparation of the v. HLA.A2.1/β-gal (O'Neil, B.H. et al J. Immunol. 151:1410-1418, 1993) v. human and murine β_2-microglobulin/β-gal, (O'Neil, B.H. et al J. Immunol. 151:1410-1418, 1993) and all the murine MHC I (L^d, D^d, K^d, and K^b)/β-gal (Restifo, N.P. et al J. Exp. Med. 177:265-272, 1993), has been previously described.

Analysis of Recombinant Proteins and Quantitation of β-galactosidase Expression

BS-C-1 monolayers were infected with 10 infectious units of recombinant virus per cell. After 20 hours infected cells were harvested in solubilization buffer (0.06M Tris-HCl (pH 6.8), 3% SDS, 5% β-mercaptoethanol, 10% glycerol and 0.002% bromophenol blue). Proteins were resolved on a 10% polyacrylamide gel and subsequently transferred to a nitrocellulose membrane using a Bio-Rad Transblott Mini cell (Bio-Rad) at 250 mAmps for 1 hour. Membranes were initially incubated for 1 hour in a blocking solution (PBS-B) consisting of PBS containing 3% non-fat milk (w/v) and 0.2% Tween 20. A murine specific B7-1 hamster antibody (supplied by Dr. H. Reiser, Dana-Farber Cancer Inst, Boston) was diluted in PBS-B and incubated with filters for 3 hours at room temperature.

Filters were subsequently washed three times in PBS containing 0.2% Tween 20. Bound primary antibodies were detected by incubating filters, after washing with ^{125}I-protein A (Amersham) at a concentration of 0.1μCi/ml in PBS-B. Filters were washed 4 times dried and exposed to X-ray film (Kodak). Protein sizes were estimated using 14C molecular weight markers (Amersham) (Fig. 13).

Recombinant viruses were analyzed for β-galactosidase expression using an enzyme assay kit (Promega, WI). Briefly, monolayers of BS-C-1 cells were infected at a multiplicity of infection (MOI) of 10. Approximately 20 hours post infection cells were washed with PBS and harvested with lysis buffer. Quantitation of enzyme expression was determined by incubating cell extracts with β-galactosidase substrate and buffer solution as described in the manufactureres protocol.

In Vivo Experiments

In vivo protection studies utilized BALB/c mice which were immunized intravenously with either Hanks's balanced salt solution (HBSS) (Biofluids, Inc., Rockville, MD), v.MCB7-1/b-gal, or v.MCMHA/b-gal (10^7plaque forming units (PFU)). Twenty-one days following this initial immunization, the mice were intravenously challenged with 5 x 10^5 tumor cells of CT26.WT or CT26.C25. Twelve days following tumor challenge, mice were randomized, euthanized, and lung metastases were enumerated in a blinded fashion.

In vivo adoptive transfer studies involved intravenous immunization of BALB/c mice with either HBSS, v.MCB7-1/b-gal or v.MC MHA/b-gal . The rVV was administered at either 10^5 or 10^7 PFUs. After twenty-one days, these mice were euthanized, and splenectomized. Following splenectomy, the spleens were morselized, passed through a Nytex membrane, and suspended in HBSS. Lymphocytes were counted on a hemocytometer, and 2 x 10^7 lymphocytes were adoptively transferred to mice who had

been injected intravenously with 5 x 10^5 tumor cells of either CT26.WT or CT26.C25. Designated groups of mice received adjuvant treatment with six doses of IL-2 administered intra-peritoneal at a dose of 100,000 Cetus units two times per day for three days. Nine days after adoptive transfer of primed splenocytes, the mice were randomized, euthanized, and lung metastases counted in a blinded fashion.

In vivo active treatment studies involved non-irradiated BALB/c mice which were inoculated intravenously with 5 x 10^5 tumor cells of either CT26.WT or CT26.C25. All mice were randomized and subsequently vaccinated with HBSS, or 10^5 or 10^7 PFUs of the designated rVV three or six days later. Designated groups of mice received adjuvant treatment with six doses of IL-2 administered intra-peritoneal at 100,000 Cetus units two times per day for three days. Mice were randomized and euthanized on day twelve, and lung metastases were counted in a blinded fashion. Identically treated groups of mice bearing three day tumor burdens were also followed long term to assess the effect of vaccination on survival.

T cell Subset Depletions

Purified culture supernatants of anti-CD4 monoclonal antibody GKI.5 (TIB 207; American Tissue Culture Collection) and ascitic fluid of hybridoma 2.43 (anti-CD8) (TIB 210: American Tissue Culture Collection) were diluted in HBSS prior to use *in vivo*. For *in vivo* depletion BALB/c mice were given i.v. injections of GK1.5 at 100 mg/ml or of empirically determined levels of 2.43 monoclonal antibodies 48 hours prior to receiving tumor challenge, and again 6 days later. Using fluorescein isothiocyante-labeled anti-CD4 and phycoerythrin labeled anti-CD8 antibodies, FACS analysis was performed 1 day prior to immunization, and again at day 7 to verify depletion.

Whole Organ X-gal Staining

Lungs from tumor-bearing mice were removed and inflated with PBS (Biofluids) prior to X-gal staining. Lungs were fixed in a solution containing 2% formaldehyde (v/v), 0.2% gluteraldehyde (v/v) in PBS for 45 minutes and washed with PBS three times. After washing, the lungs were stained with X-gal solution for 12 hours at 37° C. X-gal solution for whole organs was prepared by combining the following: 0.02% (v/v) NP-40, 0.01% (w/v) sodium deoxycolate, 1 mg/ml X-gal, 5 mM potassium ferrocyanide, 2 mM MgCl2 in PBS. After staining in X-gal solution, the lungs were rinsed briefly with 3% (v/v) dimethyl sulfate, and then with PBS and stored at 4° C in 0.02% (w/v) sodium azide in PBS.

Statistical Analysis

Data from *in vivo* protection, adoptive, and active treatment studies was analyzed using a two-sided student's *t* test for unpaired samples. Errors bars represent the standard deviation within each group. Survival was analyzed with standard Kaplan-Meier survival curves (Kaplan, E.L. J. Am. Stat. Assoc 53:457-481, 1958). All p values presented are two-sided.

Example 19

Characterization of Murine B7-1 and E.coli β-gal Genes in Cells Infected by Vaccinia Viruses

Following preparation of the rVVs, and prior to their use in *in vivo* experiments, the the *in vitro* expression of foreign protein in infected cells was evaluated by either immunohistochemical staining, western blot analysis, or both. Western blot analysis of murine B7-1 expression of virally infected cells illustrated the previously reported diffuse staining pattern typical of highly glycosylated proteins (Fig. 13) (Freeman, G.J. et al J. Exp. Med. 174:625-631, 1991). Molecular weight markers estimate VV expressed B7-1 to be approximately 55-65 k Da

in size. The authenticity of rVV expressed murine B7-1 was further verified by the binding of CTLA4-Ig to v. MCB7-1 infected cells. Expression of rVV murine B7-2 was verified by immunohistochemical staining of infected cells using a murine specific B7-2 antibody (PharMingen, CA). Expression of the TAA, β-galactosidase, was similar for v. MCB7-1/β-gal and the control recombinant v. MCMHA/β-gal, in contrast v. MCB7-1/NP showed no signs of enzyme expression (data not shown).

Example 20

Initial Screen: Ability of Immunization with Recombinant Vaccinia Virus to Mediate Regression of Established Tumor

It was determined whether combining a TAA with one or several of these immunomodulatory molecules would enhance the antigen specific immune response, as measured by the most stringent test, i.e., the ability to mediate regression of established disease. The virus, v. JS6, expressing the TAA alone, was used as control to measure either the positive or negative contribution of the immunomodulatory molecule in the vaccination vector.

The ability of a single inoculation of twelve double rVVs, which expressed the genes encoding both an immunomodulatory molecule and the β-gal model tumor antigen in the same virus, to effect regression of pulmonary metastases in tumor-bearing mice was assayed. The double rVVs were constructed to include the b-galactosidase gene within the thymidine kinase (TK) region of the VV genome for all vectors evaluated. The position of the immunomodulatory molecule for most vectors is also within the TK region of the VV genome under the same promoter as the TAA. However, in the case of the B7-1 vector, the gene for B7-1 is placed within the Hind III F region as described above and shown in Table 6.

The double rVVs studied were v.MCB7.1 (Hind III F

region of VV genome)/b-gal (TK region of VV genome), v. human b_2-microglobulin (TK)/b-gal(TK) {3154}, v. murine b_2-microglobulin (TK)/b-gal(TK) (O'Neil, B.H. et al J. Immunol. 154:1410-1418, 1993), v. murine MHC I L^d (TK)/b-gal (TK) (Restifo, N.P. J. Exp. Med. 177:265-272, 1993), v. MHC I K^d (TK)/b-gal (TK) (Restifo, N.P. J. Exp. Med. 177:265-272, 1993), v. MHC I D^d (TK)/b-gal (TK), v. MHC I (allogeneic) K^b (TK)/b-gal (TK) (Restifo, N.P. J. Exp. Med. 177:265-272, 1993), v. human leukocyte antigen (xenogeneic) HLA-A_2 (TK)/b-gal (TK) (O'Neil, B.H. et al J. Immunol. 154:1410-1418, 1993), v. murine interleukin - 2 (IL-2) (TK)/b-gal (TK), v. murine granulocyte-macrophage colony stimulating factor (GMCSF) (TK)/b-gal (TK), v. interferon-gamma (IFN-g) (TK)/b-gal (TK) (Bronte et al., in press), and v. tumor necrosis factor-alpha (TNF-a) (TK)/b-gal (TK). The v. JS6, expressing only b-gal in the TK region (described above), and a non-replicating VV, modified vaccinia Ankara (MVA) (Sutter, G. et al Proc. Nat'l Acad. Sci. U.S.A. 89:10847-10851, 1992), also only expressing b-gal, were used as controls.

Using the *in vivo* active treatment protocol outlined above, mice bearing three day pulmonary metastases were immunized with a single dose of the selected rVV. In each experiment, no therapeutic response was seen with any rVV against the parental tumor, CT26.WT, thus documenting the specificity of the response. Figure 14 displays the results of our initial screening experiment in which mice bearing three-day CT26.C25 tumors were vaccinated with rVV (10^7 PFUs).

When compared with the HBSS vaccinated control mice, only v. IL-2/b-gal and v.MCB7-1/b-gal mediated both a specific and significant reduction in the number of pulmonary metastases ($p < 0.006$ and $p< 0.002$, respectively). The ability of v. JS6, the single rVV expressing b-gal alone, to mediate significant tumor regression was variable from experiment to experiment.

(Data not shown). The inability of v. MVA/b-gal, a non-replicating VV which produces high levels of b-gal, to mediate any regression, may indicate that prolonged TAA expression (as would occur with a replicating VV) is required for a successful CTL response. Treatment with v. IL-2/b-gal and v. MCB7-1/b-gal resulted in a 10 and 5 fold further reduction in the number of metastases when compared to the best results obtained with v. JS6. These results suggest that vaccination with an immunodominant TAA alone is capable of priming a cytotoxic antitumor immune response which can then be further enhanced by the simultaneous expression of unique immunomodulatory molecules.

Although all double rVV contained the gene for the TAA, b-gal, only v. IL-2/b-gal and v. MCB7-1/b-gal were able to mediate significant reductions in the number of pulmonary metastases. Insufficient b-gal expression is unlikely to account for the therapeutic failure of the ineffective double rVVs. When tested, b-gal expression, at least between the individual cytokine producing rVV, did not significantly different (data not shown). The potential for the gene encoding for the immunomodulatory molecule within the double rVV to exert an immunosuppressive effect on the generation of a TAA cytotoxic response, or directly inhibit TAA protein expression was not evaluated, and cannot be excluded. The possibility that the failure of the GM-CSF, IFN-γ, or TNF-α producing rVVs is due to immunosuppresive effects of local cytokine production was not further explored.

Tumor regression following vaccination with v. MCB7-1/b-gal has not been previously reported. However, the therapeutic result obtained with this rVV supports the notion that costimulatory molecule expression is necessary for optimal T cell activation. Of note, a single vaccination with v. D^d/b-gal at a lower viral inoculate (10^6 PFUs) was also capable of mediating a

significant reduction in the number of pulmonary metastases ($p < 0.005$). This suggests the existence of a D^d epitope for the b-gal protein heretofore unknown.

Based on the findings of this screen, and other reports of anti-tumor effects with both B7-1 transfectants (Guinan, E.C. Blood 84:3261-3282, 1994) and v. B7-1 oncolysates (Hodge, J.W. et al Cancer Res. 54:5552-5555, 1994), a more extensively evaluate the *in vivo* effects of v. MCB7-1/b-gal on the cell-mediated antitumor immune response was conducted. Interestingly, the generation of CTL following B7-1 transfection is an IL-2 dependent phenomenon, since the addition of IL-2 blocking antibody during the induction phase blunts generation of a cytotoxic response (Harding, F.A. et al J. Exp. Med. 177:1791-1796, 1993).

Example 21

Protection Against Subsequent Tumor Challenge by Immunization with v. MCB7-1/β-gal

Although the identification of murine and human TAAs and TAA specific CTLs confirm that an immunocompetent host can generate a significant cytotoxic T cell response against autologous neoplasms; the immunogenicity of these TAA is generally weak allowing for the progressive growth of tumors. Protective immunity following vaccination with a rVV expressing either the TAA alone, or in combination with B7-1 was evaluated. The rVV strategy utilized in this current study has a distinct advantage over B7 transfections studies (in addition to the ease of administration), since the specificity of the immune response against a defined model TAA can be measured by comparing the response in the transduced tumor cell line CT26.C25, with the parental tumor, CT26.WT. Whereas the protective immunity demonstrated in the transfection studies is interpreted as a specific CTL response against an undefined TAA, the unique reactivity of the CTL in this model is easily evaluable.

Naive mice were initially vaccinated with either v. MCB7-1/b-gal, or a control virus, v. MCMHA/b-gal. The v. MCMHA/b-gal vector expressed an irrelevant protein, measles HA, which is a similarly sized and glycosylated molecule like B7-1, as well as the model TAA to allow for evaluation of the contribution of B7-1 to the response generated. Mice immunized with both v. MCB7-1/b-gal and v. MCMHA/b-gal were protected against tumor challenge with CT26.CL25, but not the wild type tumor, CT26.WT ($p < 0.0002$ and $p < 0.0014$, respectively) (Fig. 15.). These results confirm previous reports that vaccination with a rVV expressing a unique TAA alone can confer protection to subsequent challenge with a TAA-expressing tumor. (Kantor, J. et al J. Nat'l Cancer Inst. 84:1084-1091, 1992; Estin, C.D. et al Proc. Nat'l Acad. Sci. U.S.A. 85:1052-1056, 1988). The completeness of the protection by both viruses precluded a more thorough evaluation of the impact of the B7-1 gene in this situation. In this model system, b-gal protein expression by both v. MCMHA/b-gal and v. MCB7-1/b-gal was sufficient to exploit intracellular antigen processing mechanisms leading to protective immunity.

Example 22

Primary Adoptive Transfer of v. MCB7-1/β-gal Primed Splenocytes to Tumor-Bearing Syngeneic Mice

Tumorigenicity is reflective of the state of T cell activation. In the present invention it was hypothesized that vaccination a rVV coexpressing B7-1 and a model TAA, would result in the generation of a primary TAA CTL response which could be used in the adoptive therapy of tumor-bearing syngeneic host. The therapeutic advantage gained by immunizing against a known TAA to elicit CTLs reactive against both autologous or allogeneic tumors are numerous. Confirmation of the specificity of the CTL response was demonstrated in the initial observation that no reduction in the number of pulmonary metastasis was

noted after the adoptive transfer of any group of primed lymphocytes to syngeneic mice bearing CT26.WT tumors.

Mice bearing CT26.C25 tumors and inoculated with HBSS primed splenocytes showed no therapeutic benefit, however the addition of adjuvant IL-2 to similarly treated mice resulted in a significant reduction in the number of pulmonary metastases ($p < .0054$) (Fig. 16.). CT26.C25 tumor-bearing mice treated with v. MCB7-1/b-gal rVV splenocytes at two doses (10^5 and 10^7 PFUS) showed a significant reduction in tumor burden ($p < 0.007$ and $p < 0.007$, respectively). Again, adjuvant IL-2 treatment resulted in a 16.5 and 12.5 fold further reduction in the number of pulmonary metastases in this group. Significant tumor regression was also seen in mice receiving splenocytes primed with v. MCMHA/b-gal at 10^5 PFU, but not at 10^7 PFU ($p < 0.0052$, and $p < 0.094$, respectively). The addition of adjuvant IL-2 to the v. MCMHA/b-gal treatment group resulted in a 2 fold reduction in the number of pulmonary metastases in the group treated with v. MCMHA//B-gal primed splenocytes at 10^5 PFU.

Vaccination with a rVV expressing only the model TAA, was capable of priming a specific immune response which mediated tumor regression on adoptive transfer. Vaccination with a rVV containing both the costimulatory molecule, B7-1, and the model TAA, increased the therapeutic response by up to 150 fold. The increased therapeutic benefit mediated by v. MCB7-/b-gal primed lymphocytes may represent either or both a quantitative increase in the precursor frequency of TAA specific CTL in the adoptively transferred population, or a qualitative increase in the cytotoxic potential of the transferred cells mediated by B7/CD28 costimulatory signals. Infection with v. MCB7-1/b-gal would provide any MHC class I expressing cell the required elements necessary to mediate a CTL response, without the need for APCs or CD4+ T helper cells (Dohring, C. et al Int. J. Cancer 57:754-

759, 1994; Wu, Y. et al Curr. Biol. 4:499-505, 1994). Infection with v. MCB7-1/b-gal would also provide T cells with the capacity for autonomous stimulation leading to both a proliferative response to antigen and increased cytokine production (Dohring, C. et al ibid; Azuma, M. et al J. Exp. Med. 177:845-850, 1993). Notably, binding of B7/CD28 costimulates IL-2 mRNA accumulation (Linsley, P.S. et al J. Exp. Med. 173:721-730, 1991) important for T cells proliferative response, as well as IFN-γ, important in Th1 induction, as well as IL-4 important in Th2 induction (Walter, H. et al. Eur. Cytokine Netw. 5:13-21, 1994). In the absence of an antigen non-specific costimulatory signal delivered by interaction between B7-CD28, naive T cells exhibit suboptimal proliferation and decreased stability of mRNA for several cytokines important to the T cell proliferative and differentiation response (Linsley, P.S. et al Annu. Rev. Immunol. 11:191-212, 1993; Harding, F.A. et al J. Exp. Med. 177:1791-1796, 1993). Coculture of CD8+ T cells with B7+, but not B7- tumors results in potent cytotoxic response, and proliferation (Harding, F.A. et al J. Exp. Med. 177:1791-1796, 1993), which are inhibited by both anti-CD28 Fab fragments, and interestingly by anti-IL-2 antibodies. The results demonstrate that direct activation of CTL by rVV which provide appropriate costimulation and antigen specificity results in improved therapeutic response upon the adoptive immunotherapy of primed splenocytes to syngeneic tumor-bearing mice. The mechanism responsible for this response may be due to either or both a qualitative increase in antigen specific T cell precursors mediated by B7/CD28 (or CTLA-4) interaction, or B7/CD28 (or CTLA-4) mediated differentiation of T cell effector populations.

The adjuvant benefit received by adjuvant IL-2 alone upon the adoptive transfer of splenocytes in these studies confirms prior reports documenting direct IL-2 antitumor

responsiveness. The simplest explanation for the therapeutic response seen with IL-2 alone is that is represents a lymphokine activated killer cell (LAK) response mediating non-specific cytotoxicity. However, IL-2 treatment was therapeutic only in the mice bearing CT26.C25 tumors, which expressed the model TAA. Therefore, a more likely explanation for results seen with IL-2 alone and as an adjuvant, is an IL-2 mediated proliferation of antigen-specific CTL.

Example 23

Active Immunotherapy of Established Pulmonary Metastases with v. MCB7-1/β-gal

Treatment of established disease remains the ultimate goal of immunotherapy. If lack of costimulation is a principal means through which tumors evade immune surveillance in this model system, then vaccination with a v. MCB7-1/b-gal should generate an active and specific therapeutic immune response in animals bearing established tumor. To test this hypothesis, tumor-bearing mice were immunized with a single injection of rVVs, with designated groups receiving adjuvant treatment with IL-2 as outlined above. In addition to v. MCB7-1/b-gal and v. MCMHA/b-gal, v. MCB7-1/NP was used in these studies. The v. MCB7-1/NP co-expresses the B7-1 protein with an irrelevant protein (influenza nucleoprotein, NP) not expressed on the CT26.C25, and allows for assessment of the role of B7-1 expression alone. No significant tumor regression was seen following rVV vaccination in mice bearing CT26.WT tumors.

In mice bearing CT26.C25 tumors, immunization with v. MCB7-1/b-gal mediated a significant reduction in the number of metastases ($p < 0.006$) (Fig. 17.). The high therapeutic effect precluded evaluation of the effect of adjuvant IL-2. In the same experiment, mice immunized with v. MCMHA/b-gal mediated no significant tumor regression ($p < 0.36$). In four separate experiments a

significant decrease in the number of pulmonary metastases for mice immunized with v. MCMHA/b-gal at 10^7 PFU was seen only once ($p < 0.009$). IL-2 administration did not significantly improve upon tumor regression in v. MCMHA/b-gal treated mice. In the same and subsequent experiments, v. MCB7-1/NP had no effect on tumor regression. Overall, mice immunized with v. MCB7-1/b-gal showed a 170 fold and 117 fold greater reduction in the number of pulmonary metastases than mice in the v. MCMHA/b-gal and v. MCB7-1/NP immunization groups, respectively. Exogenous IL-2 alone did not mediate further tumor regression. This data implies that expression of the TAA and the costimulatory molecule are both necessary and sufficient to effect maximal regression of tumor in this active treatment model and suggests a role for B7-1 in both the induction and effector phase of the CTL response. This conclusion is further supported by the finding that a vaccination vector combining b-gal with an irrelevant protein did not reliably reduce tumor burden. (Note in one of four experiments, a significant reduction was observed, although to a much lesser degree than that seen in the v. MCB7-1/b-gal immunization group.) The presence of B7-1 alone was insufficient to effect any significant regression, which is consistent with reports that the mere expression of B7-1 on tissue without a new or foreign antigen does not result in tissue specific autoimmunity (Guerder, S. et al Immunity 1:155-166, 1994; Harlan, D.M. et al Proc. Nat'l Acad. Sci. U.S.A. 91:3137-3141, 1994).

The results from four separate active immunotherapy studies of mice immunized with 10^7 PFUs of rVV were normalized to the HBSS group for each individual experiment, and the data pooled and analyzed as percent residual disease (data not shown). Immunization with v. MCB7-1/b-gal group showed an almost 20 fold reduction in the tumor burden compared to HBSS control, with only 5.2% of tumor remaining. Adjuvant IL-2 treatment did not

improve upon this result, though a trend was seen in this group and the v. MCMHA/b-gal group. Immunization with v. MCMHA/b-gal mediated a nearly 2 fold reduction in the number of metastases with 40-55% of tumor burden remaining, while the v. MCB7-1/NP mediated no meaningful reduction with 83-93% of tumor burden remaining. This cumulative data confirms the finding seen in the individual active immunotherapy studies.

In order to evaluate whether active immunotherapy results with v. MCB7-1/b-gal were limited to the treatment of small tumor burdens, the same active treatment experiment in mice bearing six day metastases as repeated. A six day tumor burden is macroscopically visible with individual metastases measuring up to 2.5 millimeters. Again, no significant tumor regression was seen following rVV vaccination in mice bearing CT26.WT tumors. Figure 18 demonstrates that in the six day model, a single immunization with v. MCB7-1/b-gal mediated a significant reduction in the number of pulmonary metastases in mice bearing CT26.25 tumors ($p < 0.008$). Mice immunized with either v. MCMHA/b-gal or v. MCB7-1/NP had no significant reduction in the number of pulmonary metastases ($p < 0.12$, and $p < 0.22$, respectively). Again, adjuvant IL-2 did not improve this result. It is concluded therefore, that a rVV co-expressing both the costimulatory molecule, B7-1, and the TAA, b-gal, are required to mediate regression of both large and small tumor burdens in this model system. Consistent with the previous data disclosed herein and in Wang et al J. Immunol. 154(9): 4685-4692, 1995, immunization with a rVV containing only the immunodominant TAA, b-gal, was capable of inducing a cytotoxic immune response resulting in a reduction in the number of pulmonary metastases when small tumor burdens are present, but has no efficacy against a larger tumor burden. Immunization with a rVV expressing the costimulatory molecule alone without the TAA, i.e., v. MCB7-1/NP, was

unable to prime an immune response and mediate tumor regression in either situation.

A reduction in tumor burden is frequently demonstrated for several adjuvant interventions, but rarely translates into a therapeutic benefit in terms of prolonged survival. In a parallel experiment to the three day active treatment protocol described above, ten mice per group were followed daily for survival and events were recorded as either deaths, or severely moribund condition requiring euthanasia. In this study,all mice inoculated with CT26.WT were dead by day 43 with 50% dead in all groups by day 32.

CT26.C25 tumor bearing mice demonstrated varying degrees of survival among vaccination groups (Figure 19). Mice receiving HBSS, IL-2 alone, and v. MCB7-1/NP alone (with or without adjuvant IL-2), were dead by day 39. Mice vaccinated with v. MCMHA/b-gal showed a significant prolongation of survival with 50% of mice alive at day 27, and the last mouse dying at day # 46 ($p < 0.0002$) Adjuvant IL-2 did not statistically improve upon this result. In the v. MCB7-1/b-gal vaccinated mice 50 % were alive at 70 days, and 30% continue to be long term survivors at over 100 days ($p < 0.0001$). Adjuvant IL-2 therapy for this group added no statistically significant benefit to survival, though a trend was suggested with 50% still alive at day # 88, and 40% alive at 100 days follow-up.

The regression of pulmonary metastases seen following immunization with the v. MCB7-1/ b-gal thus translated into prolonged survival for most animals treated. Two mice who were long-term survivors in the v. MCB7-1/b-gal treated group, subsequently died and their lungs were subjected to whole organ X-gal staining. One of the mice showed no evidence of X-gal staining, despite bulky tumor metastases. The second mouse showed moderate positivity for X-gal staining, with some metastases exhibiting no

staining. Although the outgrowth of b-gal negative tumors as the cause of death in these long term survivors can not be concluded, it is a likely possibility.

Example 24

Active Immunotherapy of Established Pulmonary Metastases with v. B7-2/β-gal and v. MCB7-1/B7-2/β-gal

As stated earlier, optimal T cell activation require two signals, once delivered by the MHC/TCR interaction and a second from the B7-1/CD28 or CTLA-4 interaction. It was evaluated whether a rVV coexpressing B7-2 (v. MCB7-2/β-gal), or both B7-1 and B7-2 (v. MCB7-1/B7-2/β-gal), with a TAA would be a more potent vaccine to stimulate TAA specific CTL response.

Mice were initially inoculated with 5 x 10^5 tumor cells of CT26.WT or CT26.C25. Mice bearing three day tumors were then immunized with a single injection of rVV, with designated groups receiving adjuvant treatment with IL-2 as outlined above. In addition to the rVV described, an additional rVV was constructed v. MHA/B7-2/b-gal, which contained the MHA gene in the Hind III region of the VV genome, and both B7-2 and the E. coli lac Z gene in the TK region. This was created to control for the presence of the B7-1 gene in the v. MCB7-1/B7-2/β-gal construct (Fig. 20.). No significant tumor regression was seen following rVV vaccination in mice bearing CT26.WT tumors.

As seen in previous active immunotherapy experiments with mice bearing three-day CT26.C25 tumors, vaccination with v. MCB7-1/β-gal resulted in a significant reduction in the number of pulmonary metastases ($p < 0.0014$), while v. MCMHA/β-gal mediated only a minimal reduction ($p < 0.054$), and v. B7-1/NP mediated no reduction ($p < 0.13$). Vaccination with both v. MCB7-2/β-gal and v. MCB7-1/B7-2/β-gal resulted in a significant reduction in the number of pulmonary metastases ($p < 0.0018$ and $p < 0.0018$, respectively). Similarly, treatment with v. MCMHA/B7-2/β-gal also mediated significant reduction in the number of

metastases ($p < 0.0018$), which did not differ statistically from either v. MCB7-2/β-gal or v. MCB7-1/B7-2/β-gal. Adjuvant IL-2 did not statistically improve upon these results.

No statistical difference between rVV expressing either B7-1, B7-2, or both could be demonstrated. Of note, however, there is some suggestion that B7-1 may be a more potent costimulator of antitumor immunity than B7-2. That is, rVV expressing B7-1 alone mediated a 10 fold greater reduction than a rVV expressing B7-2 alone, or both B7-1 and B7-2. Similarly, rVV expressing both B7-1 and B7-2 was three times as potent as the vector containing the irrelevant protein, MHA together with B7-2. However, whereas the B7-1 and B7-2 molecules are within different regions of the VV genome, under independent promoters, whether these findings are reflective of an increased potency for B7-1 as a costimulator, or merely reflective of variable expression will require further analysis.

Example 25

Effect of In Vivo T cell Subset Depletions on the Ability of v. MCB7-1/β-gal to Mediate Tumor Regression of Established Disease

Adoptive transfer and active immunotherapy studies demonstrated a specificity of response suggesting that the cell-mediated arm of the immune system, i.e., T-lymphocytes, were responsible for tumor regression. To specifically identify the T cell subset which is responsible for the cytotoxic antitumor response observed, we repeated three-day active treatment study while selectively depleting either CD4+ or CD8+ cells with anti-CD4 and anti-CD8 antibodies. In mice treated with the anti-CD4+ antibody FACS analysis revealed > 99.8% depletion of CD4 cells, whereas in mice treated with the anti-CD8+ antibody, >97% depletion of CD8+ was seen (Figs. 21a-21f). These results were confirmed in three other

FACS analysis completed 1 day before and seven days after treatment. In the groups immunized with HBSS, no tumor regression was seen (Fig. 21g). In groups immunized with v. MCB7-1/b-gal, both the no depletion group and the CD8+ depletion group showed a significant reduction in the number of pulmonary metastases ($p < 0.005$, and $p < 0.054$, respectively). The mean reduction in the no depletion group was approximately 10 fold greater than that seen in the CD8+ depleted group. Notably, in mice depleted of CD4+ cells, there was no tumor regression seen. This suggests a critical role of CD4+ cells in mediating this cellular immune phenomenon. Similarly, in the v. MCMHA/b-gal immunized group, no reduction in the number of metastases was seen in the CD4+ depletion group, yet a small but significant reduction was seen in both the no depletion group, and the CD8+ depletion group ($p < 0.02$, and $p < 0.06$, respectively) (data not shown). Mice immunized with v. MCB7-1/NP showed no reduction in the number of pulmonary metastases in any group (data not shown). These results demonstrate that the reduction in pulmonary metastases is T cell mediated, and that both CD8+ and CD4+ lymphocytes are required to mediate this phenomenon. These results are contrary to most transfection studies in which a cytotoxic CTL response was induced in the absence of CD4+ cells. When the genes for B7-1 or B7-2 are transfected into tumor cells, it is the tumor cells themselves which act to present a TAA in the context of MHC and B7 to responding T cell. Following vaccination with a rVV coexpressing both B7 and the TAA, all infected cells expressing MHC class I molecules would theoretically function to activate T cells. In the transfection studies, CD+ 8 depletion abrogated the antitumor immune response, by removing the responding cytotoxic population. Following v. MCB7-1/b-gal vaccination however, although CD8+ depletion would significantly reduce the precursor frequency of responding

cytotoxic cells (3% remained in this study), the increased number of cells capable of presenting antigen and costimulating may blunt the effect of the depletion. Thus, the incompleteness of the CD8+ depletion may be a simple explanation for the observed results. However, the importance of CD4+ cells in providing necessary help to proliferate a CTL response in this model system cannot be minimized. CD4+ depletion completely abrogated any tumor regression following vaccination. Whether this phenomenon is mediated simply by a reduction in IL-2 production normally provided by CD4+ T helper cells, or represents a depletion of either Th1 or Th2 cells important in mediating the antitumor response is unclear.

Example 26

Materials and Methods for IL-2 Enhancement of Recombinant Poxvirus-Based Pulmonary Metastases

Cell Lines

CT26 is an N-nitroso-N-metylurethane-indiced BALB/c ($H\text{-}2^d$) undifferentiated colon carcinoma generously supplied by D. Pardoll (Baltimore, MD). CT26 was cloned to produce a wild-type parental tumor line, CT26.WT. The gene for *lacZ* was stably transfected into CT26.WT as described previously (Wang et al, J. Immunol. 154:4685, 1995). Briefly, a plasmid donated from A.D. Miller (Fred Hutchinson Cancer Research Center, Seattle, WA) containing the gene for β-gal and a neo-resistance marker were used to construct an ecotropic producer cell line secreting the LZSN retroviral construct. This retroviral supernatant was used to transduce the CT26.WT cell line. Transductants were selected in a G418 media and then subcloned by limiting dilution analysis at 0.3 cells/well. Subclones that expressed β-gal were screened by X-gal staining and in ^{51}Cr release assays with anti-β-gal effectors. The subclone CT26.CL25 was selected for use in all studies because of its stable expression of both β-gal and the class I molecule $H\text{-}2\ L^d$. A clone of the mouse

thymoma EL4 (H-2^b) stably transfected with β-gal, termed E22 (provided by Y. Paterson, (Department of Microbiology, University of Pennsylvania, Philadelphia) was used as a negative control in ^{51}Cr release assays. BSC-1 cells (American Type Culture Collection (ATCC), Rockville, MD; CCL 26) were used to expand and titer all VV. Cell lines were maintained in RPMI 1640, 10% heat-inactivated FCS (Biofluids, Rockville, MD), 0.03% L-glutamine, 100 μg/ml streptomycin, 100 μg/ml penicillin, and 50 μg/ml gentamicin sulfate (National Institutes of Health Media Center, Bethesda, MD). CT26.CL25 and E22 were maintained in media containing 400 μg/ml G418 (Life Technologies, Inc. Grand Island, NY).

rVV and rFPV

All the rVV used in this study were originated by insertion of the foreign genes into the VV thymidine kinase (TK) gene by homologous recombination, resulting in the generation of TK-negative progeny as described (Chakrabarti et al, Mol. Cell Biol. 5:3403, 1985). The recombinant stocks were produced by using the TK-human osteosarcoma 143/B cell line (ATCC, CRL8303). From these stocks, rVV were propagated in BSC-1 cells and used as crude cell lysate. The BSC-1 cell line was also utilized to determine virus concentration by plaque titration. The rVVV used in a single experiment were titered together to maximize the accuracy of the relative titers. Preparation of rVV expressing the influenze A/PR/8/34 nucleoprotein (NP) was previously described (Smith, G.L. et al Virology 160:336, 1987). In the HPV16-E6 Vac, *Escherichia coli lacZ* was under the control of the early promoter element of the VV $p_{7.5}$ promoter from plasmid pSC65 (S. Chakrabarti, J. Sisler, and B. Moss, NIAID, NIH, Bethesda, MD); this construct was named VJS6 for simplicity. Control CR19 VV (wild-type vaccinia) was kindly provided by J. Yewdell and J. Bennink (NIAID, NIH, Bethesda, MD). Murine IL-2 cDNA

was amplified by PCR from pBMGNomIL-2 and ligated into the *SmaI-BamHI* site of a vaccinia expression vector, pMJ601 (a gift from B. Moss), which contains the β-gal gene under the control of the $p_{7.5}$ early vaccinia promoter (Whitman, E.D. et al, Surgery 116:183, 1994). The other cytokines (GM-CSF, IFN-γ, and TNF-α) were inserted into the wtVV genome with a similar procedure. The cytokines produced after infection with the rVV have been confirmed for their bioactivity.

The POXVAX-TC strain of FPV was used in these studies and is designated FPV.wt. Foreign sequences were inserted into FPV by homologous recombination as described by Jenkins, S.L. et al, AIDS Res. Hum. Retroviruses 7:991, 1991. FPV.bg40K is a recombinant that contains the *E. coli lacZ* gene under the control of the VV 40k promoter, placed in the *BamHI* J region of the FPV genome.

Peptides

The synthetic peptide, TPHPARIGL, spanning amino acids 876-884 of β-gal, the naturally processed H-2 L^d-restricted epitope (Gavin, M.A. et al, J. Immunol. 151:3971, 1993), was synthesized by Peptide Technologies (Washington, D.C.) to a purity of greater than 99% as determined by HPLC and amino acid analysis.

Evaluation of primary response

Primary lymphocyte populations were obtained by injecting 8- to 12-wk-old female BALB/c mice (Animal Production Colonies, Frederick Cancer Research Facility, National Institutes of Health, Frederick, MD) i.v. with varying PFUs of recombinant viruses. The spleens were taken on day 6 after immunization, separated into a single cell suspension and tested for their ability to lyse β-gal-positive targets in a 6 $h^{51}Cr$ release assay. Splenocytes were resuspended in complete media including RPMI 1640 with 10% FCS (Biofluids, Rockville, MD), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate (Biofluids) and 5 x 10^{-5} μM2-ME (Life Technologies, Inc., Rockville,

MD), and used in 6-h_{51}CR release assays as described elsewhere (Restifo, N.P. et al J. Immunol. 147:1453, 1991). To summarize, 2 x 10^6 target cells were treated with 200 mCi $Na^{51}CrO_4$ (^{51}Cr) for 90 min. Peptide-pulsed CT26.WT were incubated with 1 μg/ml of peptide during labeling. Target and effector cells were mixed at appropriate ratios for 6 h. The amount of ^{51}Cr released was determined by gamma-counting and the percent specific lysis was calculated from triplicate samples according to the following formula:

[(experimental cpm - spontaneous cpm)/
(maximal cpm - spontaneous cpm] x 100.

Detection of cytokine production

Cytokine production was determined by ELISA. The ELISA kits for detection of murine GM-CSF, IFN-γ, TNF-α, and IL-2 from Endogen (Endogen, Boston, MA) were used according to the manufacturer's instructions. IL-2 concentrations were sometimes given in Cetus units, 1 Cetus unit/ml corresponding to 600 pg/ml of IL-2.

In vivo treatment studies

BALB/c mice were immunized with virus (5 x 10^6 to 10^7 PFUs) 3 or 6 days after i.v. challenge with tumor cells (10^5 to 5 x 10^5) to establish pulmonary metastases. All animals were randomized before receiving virus. Treatment with IL-2 was initiated 12 h after immunization; 6 doses of high dose IL-2 (100,000 Cetus units/injection) or 10 doses of low dose IL-2 (15,000 Cetus units/injection) were administered to selected groups of mice. Mice were killed on day 12 and lung metastases were enumerated in a blind fashion. Identically treated groups of mice were followed for survival.

Whole organ X-gal staining

Lungs from mice treated with i.v. tumor challenge as described above were inflated with PBS (Biofluids) before X-gal staining. Lungs were fixed in a solution containing

2% formaldehyde (v/v), 0.2% glutaraldehyde (v/v) in PBS for 45 min, washed in PBS three times, and stained in X-gal solution for 12 h at 37°C. X-gal solution for whole organs was prepared by combining the following: 0.02% (v/v) Nonidet P-40, 0.01% (w/v) sodium deoxycholate, 1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$ in PBS. After staining in X-gal solution, the lungs were rinsed briefly with 3% (v/v) dimethyl sulfate, and then with PBS. The stained lungs were stored at 4°C in 0.02% (w/v) sodium azide in PBS (Lin, W.C. et al, Cancer Res. 50:2808, 1990).

Statistical analysis

The Wilcoxon-Mann-Whitney U test was used to examine the null hypothesis of identify of ranks between two sets of data. Kaplan-Meier plots and Mantel-Haenszel test were used to compare survival of mice belonging to different treatment groups.

Example 27

Administration of Exogenous IL-2 with rVV encoding the model TAA reduces the number of pulmonary metastases and prolongs survival

When inoculated i.v. at a dose of 5×10^5 cells, both the parental line CT26.WT and the subclone CT26.CL25, transduced with a retrovirus encoding the model TAA β-gal, grew progressively and killed the animals in 11 to 15 days. At the time of death, these mice had greated than 500 pulmonary metastases. In the experiment presented in Table 8, control mice received no treatment and had pulmonary metastases that were too numerous to count. Neither the inoculation of moderate doses of rIL-2 for 5 days nor one i.v. injection of 5×10^6 PFU/mouse of a rVV-encoding β-gal (VJS6) induced a significant reduction of the number of pulmonary metastases counted 12 days after tumor inoculation. Immunization with VJS6 3 days after tumor injection combined with rIL-2 administration induced a significant reduction in CT26.CL25 pulmonary metastases

(p = 0.005) whereas no significant decrease in the number of pulmonary metastases of the TAA-negative parental line was seen.

Table 8

Treatment of Established Pulmonary Metastases with rVV and exogenous IL-2[d]

	Mice Inoculated With			
	CT26.WT		CT26.CL25	
rVV Treatment	Average no. metastases	Metastases/Mouse	Average No. metastases	Metastases/Mouse
None[b]	>500	>500 x 5	>500[c]	>500 x 5
VJS6	>500	>500 x 5	405.2	>500 x 3, 267, 259
Exogenous rIL-2	>500	>500 x 5	>500	>500 x 5
Exogenous RIL-2 + VJS6	406.4	>500 x 3, 298, 234	20.8	43, 6, 0, 52, 3

[a] Five BALB/c mice per each treatment group were injected i.v. with 0.5 ml of HBSS containing 5 x 10^5 tumor cells of either CT26.WT or CT26.CL25. Three days later they received a single i.v. injection of 5 x 10^6 PFU of β-gal expressing rVV, VJS6. Treatment with exogenous rIL-2 (15,000 Cetus U, twice a day, i.p.) was started 6 h after rVV inoculation and continued for 5 days. Lungs were harvested on day 12 after tumor inoculation and pulmonary metastases were counted in a blind fashion.

[b] Control mice were injected with HBSS alone.

[c] All the mice in this group died between days 11 and 12 (before lung harvest).

Active immunotherapy with the combination of exogenous rIL-2 and rVV also prolonged the survival of mice bearing 3-day-old pulmonary metastases (Figs. 22a and 22b). Two administration regimes for IL-2 were chosen: 100,000 U rIL-2 for 3 days (high dose) and 15,000 U for 5 days (low dose) were administeredd i.p. following a single i.v. injection of VJS6. As an additional negative control, a group of mice was treated with the highest dose of exogenous rIL-2 together with a TK-disrupted rVV expressing a protein different from β-gal, the influenze virus NP (V69; Figs. 22a and 22b). VJS6 exerted a marginal effect on mouse survival, consistent with a partial reduction of the number of metastases observed in some experiments (see below). A clear improvement of

survival was obtained when exogenous rIL-2 was administed with the β-gal-encoding rVV but not with the NP-expressing rVV (Fig. 22b). There was no significant difference in the survival of mice receiving the "high dose" or "low dose" rIL-2 regimens (p = 0.231). Improved survival of mice bearing the parental cell line was not observed in any treatment group (Fig. 22a).

The therapeutic advantage of exogenous rIL-2 was not limited to fully replication-competent viruses because a prolongation of survival was also observed with rFPV encoding β-gal but not with the wild-type virus (Figs. 23a and 23b). The effect of a single i.v. injection of 10^7 PFU of the recombinant FPV.bg40k, expressing β-gal under the control of the 40k vaccinia promoter, on the survival of mice bearing the β-gal-positive tumor was limited and consistent with a partial reduction in the number of pulmonary metastases. However, daily inoculation of high IL-2 doses significantly increased the therapeutic effect, resulting in 40% of mice still surviving 2 mo after treatment.

To examine a more advanced disease model, mice were injected with 10^5 tumor cells and treated on day 6. Following the i.v. inoculation of 10^5 CT26.CL25 tumor cells, all the untreated mice died by day 22 (Fig. 24). Lungs examined after 6 days of tumor growth revealed the presence of more than 100 macroscopically visible nodules (not shown). At this time point, a single inoculation of VJS6 was able to slightly increase survival, but all the mice died within 24 days. Addition of high rIL-2 dose treatment resulted in a significant survival benefit (p = 0.005) also in this advanced disease model, with two of five mice surviving until day 35. Once again, the response was specific and limited to the combination of β-gal-positive tumor and rVV; the survival of CT26.WT tumor-bearing mice was not affected (data not shown).

Example 28

Therapeutic efficacy of IL-2 produced from drVV also expressing the model TAA

The large vaccinia genome can host up to 25 kb of exogenous DNA, allowing the expression of different heterologous proteins in addition to the model TAA (Moss, B. Curr. Opin. Genet. Dev. 3:86, 1993; Mackett, M. et al. Proc. Natl Acad. Sci. USA 79:7415, 1982). The results obtained with exogenous rIL-2 prompted us to investigate the effect of cytokine production at the site of virus infection and replication. A drVV co-encoding the model TAA β-gal and IL-2 was previously described (Flexner, C. et al, Vaccine 8:17, 1990). On the basis of the above results with passively administered IL-2, a new set of dr VV in which cytokine production was driven by a powerful synthetic promoter (Davison, A.J., Nucleic Acids Res. 18:4285, 1990) was constructed. The murine cytokines included in this study were the following: IL-2, GM-CSF, IFN-γ, and TNF-α. Because the same plasmid was used to construct the various TK⁻drVV, a similar level of β-gal enzymatic activity was detected after infection of the BSC-1 cell line (data not shown). Supernatants from the same infected cultures were harvested at various times and tested for the presence of the different cytokines. Table 9 shows that specific and elevated cytokine production was detected only in supernatants from BSC-1 cells infected with the relevant viruses. For example, approximately 40,000 U IL-2/ml were released during 36 h in supernatant from BSC-1 at a moi of 1. IL-2 was barely detectable in all the other supernatants. Comparable levels of IL-2 production in murine and human tumor cell lines infected with the same rVV were recently reported (Whitman, E.D. Surgery 116:183, 1994). No cytokine was present in supernatants from cell cultures infected with the VJS6 virus expressing the E6 protein from HPV16 in addition to β-gal.

Table 9

Detection of cytokine production following in vitro infection of BSC-1 cells with 1:1 moi of different rVV[a]

Cytokine production after 12 h				
rVV	IL-2	GM-CSF	IFN-γ	TNF-α
VJS6	<34	<15	<47	<30
IL-2	2×10^6	<15	<47	<30
GM-CSF	<34	5.1×10^5	<47	<30
IFN-γ	<34	<15	5.6×10^4	<30
TNF-α	<34	<15	<47	2.05×10^3
Cytokine production after 36 h				
VJS6	<34	<15	<47	<30
IL-2	3.9×10^6	<15	<47	<30
GM-CSF	54	4.2×10^6	<47	<30
IFN-γ	94	<15	6.7×10^4	<30
TNF-α	<34	2.2×10^{3b}	<47	1.35×10^5

a. Duplicate wells of 10^5 BSC-1 cells were infected with different rVV at a multiplicity of infection (moi) of 1 PFU/cell. At different intervals, supernatants were removed, centrifuged to eliminate cellular debris, serially diluted, and used to estimate cytokine production by using an ELISA assay specific for the murine cytokines indicated in the table. Values are expressed as pg/ml of supernatant.

b. Only one of the two wells was positive. No positivity was detected in subsequent determinations.

When a single i.v. injection of 5×10^6 PFU/mouse was used to treat mice bearing 3-day-old pulmonary metastases, only the IL-2 drVV was able to significantly reduce the number of pulmonary nodules in mice inoculated with the β-gal-positive tumor cell line ($p = 0.005$) (Fig. 25b). A partial decrease was obtained with the other cytokine-encoding viruses and with VJS6. No appreciable change was observed in the number of metastases of the parental tumor cell line (Fig. 25a). The effect on pulmonary metastases also correlated with an increased survival (see below).

These resuls were particularly interesting because the drVV expressed 20- to 50-fold less β-gal enzyme activity than the VJS6 rVV, as detected after infection of BSC-1 cells with an equivalent moi (data not shown) and

also suggested that the production of IL-2 could overcome the lower production of the model TAA. When compared under the same experimental conditions, IL-2 drVV together with exogenous IL-2 gave the best results in terms of prolongation of survival ($p = 0.002$), whereas the survival in the group treated with IL-2 rVV or the combination VJS6 plus exogenous IL-2 was similar (Figs. 26a and 26b).

Example 29

IL-2 enhances the primary CTL response against VV and heterologously expressed β-gal

Because the presence of $CD8^+$ lymphocytes recognizing tumor determinants has been associated with an antitumor effect (Greenberg, P.D., Adv. Immunol. 49:281, 1991), the generation of effector CTL against β-gal in the spleens of mice 6 days after immunization was studied. This time point represents the peak of the $CD8^+$ CTL anti-vaccinia response (Bennink, J.R., Curr. Top. Microbiol. Immunol. 163:153, 1986). BALB/c mice were immunized with different rVV indicated in Figure 27, and the spleens of two mice, removed 6 days after immunization, were pooled and tested directly in a short term ^{51}Cr release assay. The response of VJS6-immunized splenocytes against the CT26.WT target cells infected with the crude 19 VV can be assumed to be the baseline for the primary response elicited by a TKVV, because the viruses presented in Figure 27 were all generated through recombination in the TK region of the vaccinia genome. The virus-driven cytokine production resulted in either an increase (IL-2 and GM-CSF rVV) or in a suppression (TNF-α and IFN-γ) of the primary cytotoxic response to vaccinia Ags. This effect was even more pronounced when a comparison between the lytic units in each spleen was performed because the difference in spleen size among the different groups; in fact, the number of lytic units in GM-CSF and IL-2 groups were increase 8.3- and 6.4-fold, whereas TNF-α and IFN-γ were reduced 5.1- and 2.9-fold, respectively, compared with the control

cytokine-negative VJS6 (Fig. 27). In the same experiments, the primary response against β-gal-positive target cells was evaluated but no lysis was detected, with the exception of a small response (5 to 10% specific ^{51}Cr release) in mice immunized with IL-2 drVV (see below). After in vitro restimulation with the antigenic peptide from β-gal (TPHPARIGL), splenocytes of BALB/c mice immunized 14 days before with 5 x 10^6 PFU of different drVV generated effector CTL able to specifically recognize the β-gal-positive clone CT26.CL25 or the parental cell line pulsed with the minimal determinant antigenic peptide. Despite the clear effect of IL-2, either exogenously administered or endogenously produced by rVV, in the treatment of tumor-bearing mice, no difference in the cytolytic activity against the specific target cells was observed in secondary cultures from animals immunized with different rVV co-expressing the TAA with the different murine cytokines (data not shown).

Because analysis of primary and secondary responses did not show any clear difference in reactivity against β-gal in normal mice inoculated with different rVV, the possibility that the in vivo response could be influenced by the presence of growing tumor by comparing the primary cytotoxic activity in normal and in tumor-bearing mice was investigated. Groups of three mice were mock inoculated (HBSS) or injected with different doses (5 x 10^5, 10^5, 5 x 10^4) of either the parental CT26.WT or the β-gal-positive CT26.CL25 cell line. After 3 days mice received a single i.v. dose of different rVV, and their spleens, collected after 6 days from virus immunization, were tested for primary CTL activity. Figure 28 summarizes the results of one experiment representative of three others. As previously indicated, immunization of normal BALB/c mice with IL-2 drVV produced a small but consistent primary response against the β-gal peptide-pulsed CT26.WT and the transduced CT26.CL25 target cells; this response was

augmented in a dose-dependent fashion in mice bearing an increasing number of β-gal-positive pulmonary metastases as a consequence of an increased inoculum of tumor cells. Conversely, the specific anti-β-gal response in mice injected with the highest dose of CT26.WT tumor cells was not comparably increased (in Fig. 28 the mice inoculated with the highest number of CT26.WT cells were shown) and was characterized by an elevated level of nonspecific cytotoxicity against the E22 target cells, a β-gal-positive line expressing a different restriction element ($H\text{-}2^b$). No response was observed in mice inoculated with 5×10^5 CT26.CL25 cells and treated with VJS6 (Fig. 28) or drVV expressing GM-CSF, IFN-γ, or TNF-α (now shown). The same enhancement of primary response was obtained with exogenous IL-2 and VJS6 (data not shown).

Example 30

Pulmonary metasases in mice dead after a prolonged survival do not present detectable levels of the model TAA

Tumor cells devoid of the β-gal marker protein are easily detected by a simple assay, facilitating the recognition of antigenic modulation occurring in vivo. Lungs from mice found dead during the survival follow-up were inflated, fixed, and exposed to an X-gal solution allowing the staining of the whole organ (Lin. W.C. et al, Cancer Res. 50:2808, 1990). Lungs from untreated mice, dead after 12 to 16 days from the i.v. injection of 5×10^5 CT26.CL25 tumor cells, showed an intense, dark-blue staining. Lungs removed from mice treated with the combination high dose exogenous rIL-2 and rVV and found dead after 50 days of survival in the experiment described in Figs. 22a and 22b were completely negative for X-gal staining as were the mice inoculated with the β-gal-negative parental cell line (data not shown). The only slight positivity detected in one sample was associated with the mouse liver, strongly adherent to the lung through a β-gal-negative invasive metastasis. This

positivity is probalby related to the β-gal-like activity present in lysosomal compartment (Sanes, J.R. et al, EMBO J. 5:3133, 1986)). A similar negativity to X-gal staining was observed in pulmonary metastases from mice treated with the combination IL-2-rVV and exogenous rIL-2 in the survival study presented in Figs. 26a and 26b.

Example 31

The effects of recombinant IL-10 on the therapeutic effectiveness of replicating and non-replicating poxviruses

BALB/c mice (5-10/group) were injected intravenously with 5 x 10^5 CT26-β-gal transfectants (CT-26.C.25) then randomized. Three days later, mice were treated with a recombinant vaccinia virus (designated VJS6) that encodes the model tumor antigen (full-length β-galactosidase) at 10^6 and 10^7 PFU either alone or in combination with 0.1μg of IL-10 I.P. per day for 7 days starting 12 hrs after the virus was given. The source of IL-10 was human recombinant IL-10 obtained from Biological Response Modifiers Program (Frederick, MD). As designated, other groups of mice were treated with a non-replicating recombinant fowlpox virus that also encodes the model tumor antigen (full-length β-galactosidase) at 10^6 and 10^7 PFU either alone or in combination with 0.1μg of IL-10 I.P. per day for 7 days starting 12 hrs after the virus was given. Pulmonary metastases were enumerated on day 12 in a coded, blinded fashion. The data is provided in Table 10.

Table 10

Adjuvant Therapy in a replicating and non-replicating setting

Group	Avg # Mets	Breakdown	P2
No Tx	250	tntc*5	---
VJS6 10e6	198	tntc*3,135,95,208	.05 (vs. no tx)
VJS6 10e6 + IL10	92	45,70,85,74,112,125,130,90	.0036 (vs. VJS6)
VJS6 10e7	49	44,40,41,84,41,40,55	.0028 (vs. no tx)
VJS6 10e7 + IL10	28	52,35,3,20,31	.005 (v. VJS6)
IL-10 alone	250	tntc*5	---
FPV 10e6	83	84,89,40,94,84,65,49,61,125	.002 (vs. no tx)
FPV 10e6 + IL10	20	15,4,6,8,0,12,40,82	.024 (v. FPV)
FPV 10e7	10	0*2,3,3,5,2,15,12,49	.002 (vs. no tx)
FP 10e7 + IL10	24	0,0,2,4,29,30,55,38,45,46	>.05

In another experiment BALB/c mice (5-10/group) were injected intravenously with 5 x 10^5 CT26-β-gal transfectants (CT-26.C.25) then randomized. Three days later, mice were treated with number of PFU listed on the x-axis of a recombinant vaccinia virus (designated VJS6) that encodes the model tumor antigen-full-length β-galactosidase either alone or in combination with 0.1μg of IL-10 per day for 7 days starting 12 hrs after the virus was given. Pulmonary metastases were enumerated on day 12 in a coded, blinded fashion. Animals receiving no treatment or IL-10 alone had an average of 250 pulmonary metastases (Figure 29).

In yet another experiment, mice were injected I.V. with 5 x 10^5 CT26-β-gal transfectants. Three days later mice were treated with 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, or 10^7 PFU of VJS6 either alone or in combination with 0.1μg of

IL-10 per day for 7 days starting 12 hrs after the virus was given. Pulmonary metastases were enumerated on day 12 as above. Animals receiving no treatment or IL-10 alone had an average of 250 pulmonary metastases (Figure 30).

While the invention is described above in relation to certain specific embodiments, it will be understood that many variations are possible, and that alternative materials and reagents can be used without departing from the invention. In some cases such variations and substitutions may require some experimentation, but will only involve routine testing.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without departing from the generic concept, and therefore such adaptations and modifications are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments.

All references and patents referred to are incorporated herein by reference.

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What is claimed is:

1. A recombinant virus comprising a recombinant virus which has incorporated into a viral genome or portion thereof one or more genes or portion thereof encoding an antigen of a disease causing agent and one or more genes or portions thereof encoding an immunostimulatory molecule.

2. The recombinant virus according to claim 1 where the virus is nonpathogenic.

3. The recombinant virus according to claim 1 wherein the virus is nonreplicating.

4. The recombinant virus according to claim 1 wherein the virus is selected from the group consisting of retrovirus, baculovirus, Ankara virus, fowlpox, adenovirus, and vaccinia virus.

5. The recombinant virus according to claim 1 wherein the disease causing agent is a cancer or pathogenic microorganism.

6. The recombinant virus according to claim 5 wherein the cancer is a non-Hodgkin Lymphoma, leukemia, Hodgkins lymphoma, lung cancer, liver cancer, metastases, melanoma, adenocarcinoma, thymoma, colon cancer, uterine cancer, breast cancer, prostate cancer, ovarian cancer, cervical cancer, bladder cancer, kidney cancer, pancreatic cancer or sarcoma.

7. The recombinant virus according to claim 5 wherein the pathogenic microorganism is virus, bacterium, protozoan, or yeast.

8. The recombinant virus according to claim 7

wherein the pathogenic virus is HIV, hepatitis virus, human papillomavirus, equine encephalitis virus, herpes simplex virus or influenza virus.

9. The recombinant virus according to claim 6 wherein the antigen is a tumor associated antigen.

10. The recombinant virus according to claim 9 wherein the tumor associated antigen is selected from the group consisting of oncofetal antigens, MART-1, Mage-1, Mage-3, gp100, tyrosinase, CEA, PSA, CA-171A, CA-19-A, CA-125, erb-2, TRP-1, P-15 and β-galactosidase.

11. The recombinant virus according to claim 1 wherein the immunostimulatory molecule is selected from the group consisting of IL-2, B7.1, B7.2, ICAM-1, LFA-3, CD72, GM-CSF, TNFα, IFNγ, IL-10, IL-12, IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-13, IL-14, IL-15, ICAM-2, LFA-1, G-CSF, M-CSF, IFNα, CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MGSA, MIP-1α, MIP-1B, RANTES and combinations thereof.

12. The recombinant virus according to claim 11 comprising the immunostimulatory molecule, IL-2 and B7.1.

13. The recombinant virus according to claim 1 wherein the recombinant virus is vaccinia virus.

14. The recombinant virus according to claim 13 wherein the gene encoding the antigen is incorporated into a HIND F13L region or TK region of the vaccinia virus.

15. The recombinant virus according to claim 13 wherein the gene encoding the immunostimulatory molecule is incorporated into a HIND F13L region or HA region of the vaccinia virus.

16. A recombinant virus comprising a recombinant vaccinia virus which has incorporated into a genome a gene encoding a tumor associated antigen and a gene encoding an immunostimulatory molecule.

17. The recombinant virus according to claim 16 wherein the tumor associated antigen is an oncofetal antigen, MART-1, Mage-1, Mage-3, gp100, tyrosinase, CEA, PSA, CA-171A, CA-19-A, CA-125, erb-2, TRP-1, P-15 or β-galactosidase.

18. The recombinant virus according to claim 16 wherein the immunostimulatory molecule is ICAM-1, LFA-3, CD72, GM-CSF, TNFα, IFNγ, IL-12, RANTES, IL-10, IL-2, B7.1, B7.2, IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-13, IL-14, IL-15, ICAM-2, LFA-1, G-CSF, M-CSF, IFNα, CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MGSA, MIP-1α, MIP-1B or combinations thereof.

19. A method of preventing a disease in a mammal comprising administering to the mammal an effective amount of a recombinant virus according to claim 1, alone or in combination with an exogenous immunostimulatory molecule said amount is effective in preventing or ameliorating said disease.

20. The method according to claim 19, wherein the disease is caused by a cancer or pathogenic microorganism.

21. The method according to claim 19, wherein the cancer is non-Hodgkin lymphoma, Hodgkins lymphoma, leukemia, lung cancer, liver cancer, metastases, melanoma, adenocarcinoma, thymoma, colon cancer, uterine cancer, breast cancer, prostate cancer, ovarian cancer, cervical cancer, bladder cancer, kidney cancer, pancreatic cancer or sarcoma.

22. The method according to claim 19 wherein the pathogenic microorganism is an intracellular virus, bacterium, protozoan, or yeast.

23. The method of claim 19 wherein the exogenous immunostimulatory molecule is IL-2, GM-CSF, TNFα, TNFγ, IL-12, RANTES, B7-1, B7-2, ICAM-1, LFA-3, IL-10, CD72, IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-13, IL-14, IL-15, ICAM-2, LFA-1, G-CSF, M-CSF, IFNα, CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MGSA, MIP-1α, MIP-1B or combinations thereof.

24. A pharmaceutical composition comprising the recombinant virus according to claim 1 alone or in combination with an exogenous immunostimulatory molecule, chemotherapy drug, antibiotic, antifungal drug, antiviral drug or combination thereof and a pharmaceutically acceptable carrier.

25. The pharmaceutical composition according to claim 24 wherein the exogenous immunostimulatory molecule is ICAM-1, LFA-3, CD72, GM-CSF, TNFα, IFNγ, IL-10, IL-12, RANTES, IL-2, B7.1, B7.2, IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-13, IL-14, IL-15, ICAM-2, LFA-1, G-CSF, M-CSF, IFNα, CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MGSA, MIP-1α, MIP-1B or combination thereof.

26. A method of making an immune-enhancing recombinant virus against a disease causing agent comprising inserting a nucleic acid sequence encoding one or more antigens or immunodominant epitopes thereof and a nucleic acid sequence encoding one or more immunostimulatory molecules into the genome or portion thereof of a recombinant virus.

27. An immune-enhancing recombinant virus made

according to the method of claim 26.

28. A pharmaceutical composition comprising a recombinant virus, one or more exogenous immunostimulatory molecules and a pharmaceutically acceptable carrier, said virus having incorporated into a viral genome or portion thereof one or more genes or portion thereof encoding an antigen of a disease causing agent.

29. The pharmaceutical composition according to claim 28 wherein the disease causing agent is a cancer or a pathogenic microorganism.

30. The pharmaceutical composition according to claim 28 wherein the antigen is a tumor associated antigen.

31. The pharmaceutical composition according to claim 28 wherein the exogenous immunostimulatory molecule is IL-2, GM-CSF, TNFα, IFNγ, IL-10, IL-12, RANTES, B7.1, B7.2, ICAM-1, LFA-3, IL-10, CD72, IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-13, IL-14, IL-15, ICAM-2, LFA-1, G-CSF, M-CSF, IFNα, CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MGSA, MIP-1α, MIP-1B or combination thereof.

32. The pharmaceutical composition according to claim 30 wherein the exogenous immunostimulatory molecule is IL-2, IL-10, or combination thereof.

33. A method of preventing a disease in a mammal comprising administering to the mammal an effective amount of the composition according to claim 28, said amount is effective in preventing or ameliorating said disease.

1/32
105
137

FIG. 1

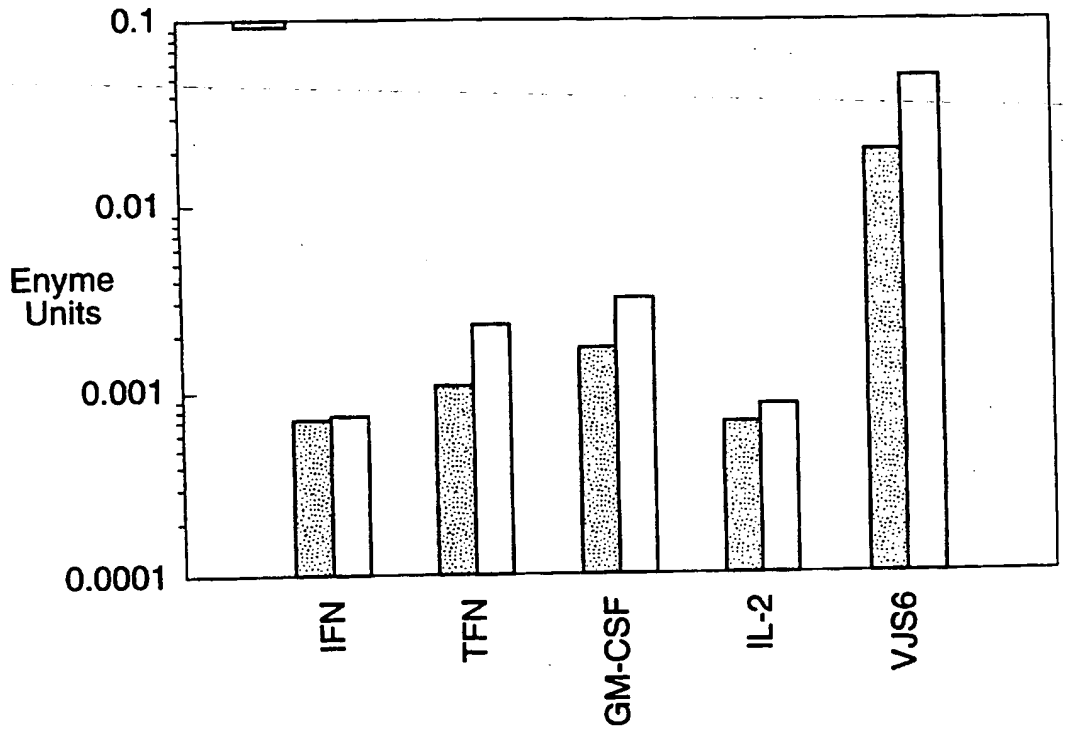


FIG. 2

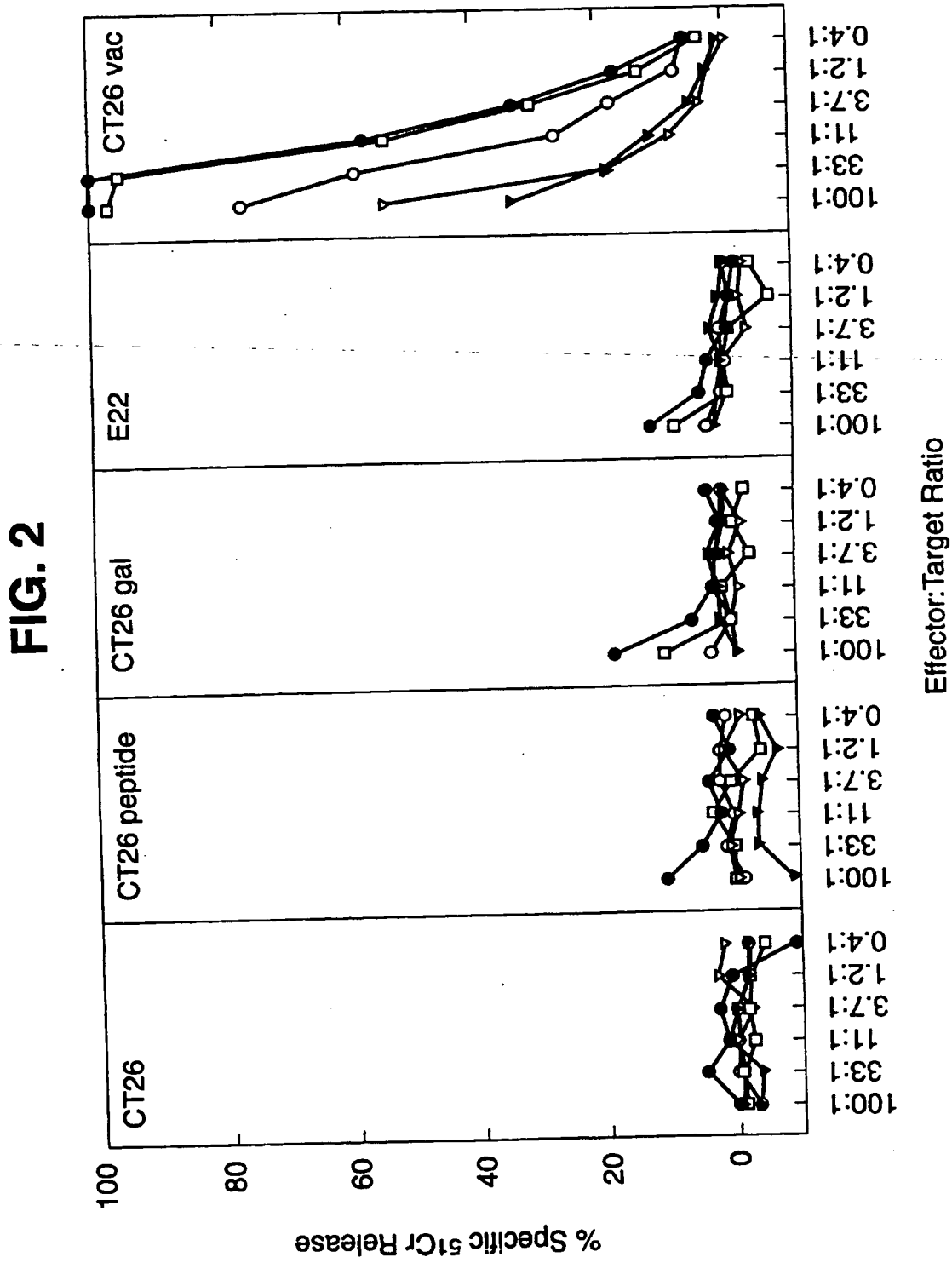

CT26 vac
E22
CT26 gal
CT26 peptide
CT26
100:1
33:1
11:1
3.7:1
1.2:1
0.4:1
Effector:Target Ratio
% Specific 51Cr Release
100
80
60
40
20
0

FIG. 3

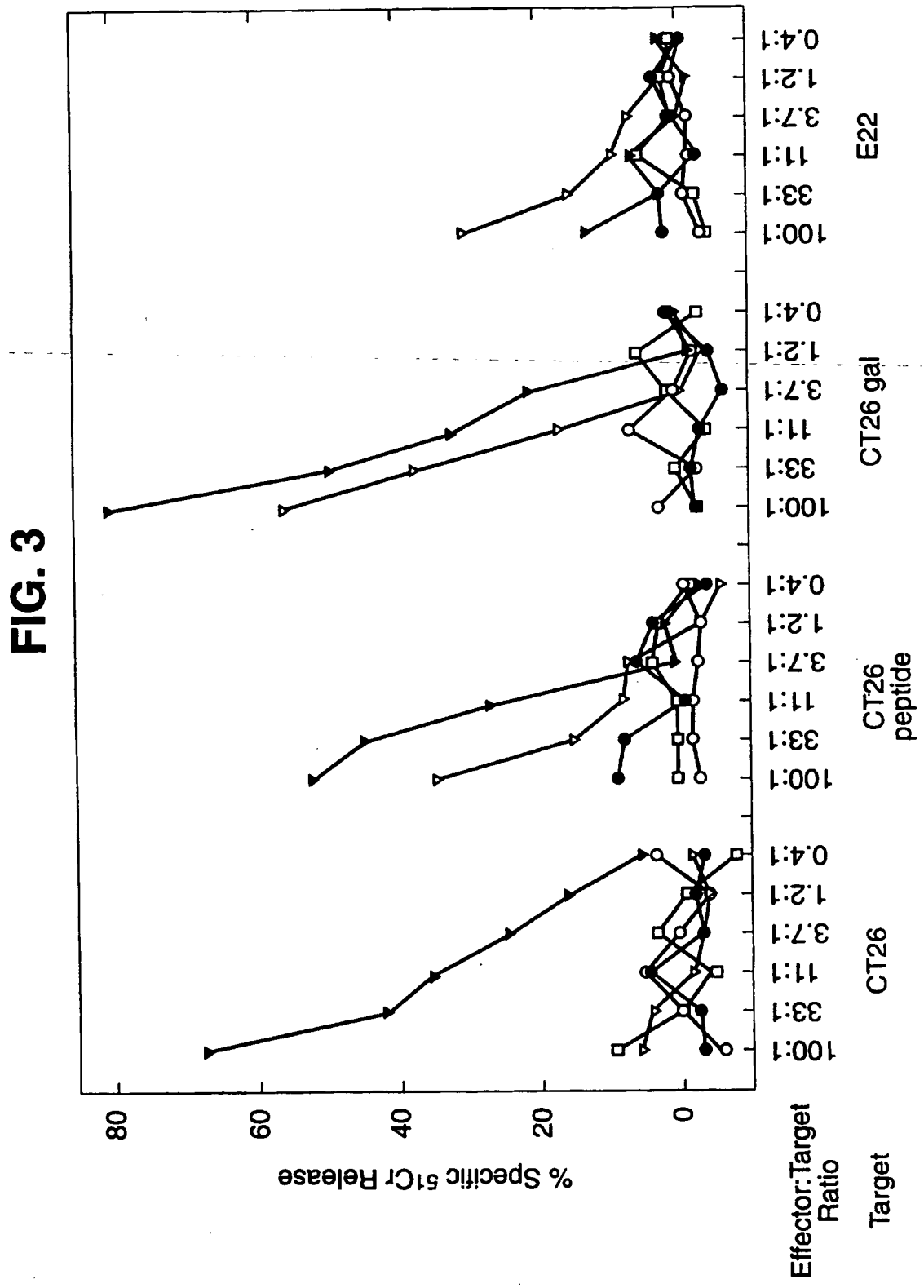

% Specific 51Cr Release
0
20
40
60
80
Effector:Target Ratio
Target
100:1
33:1
11:1
3.7:1
1.2:1
0.4:1
CT26
CT26 peptide
CT26 gal
E22

FIG. 4

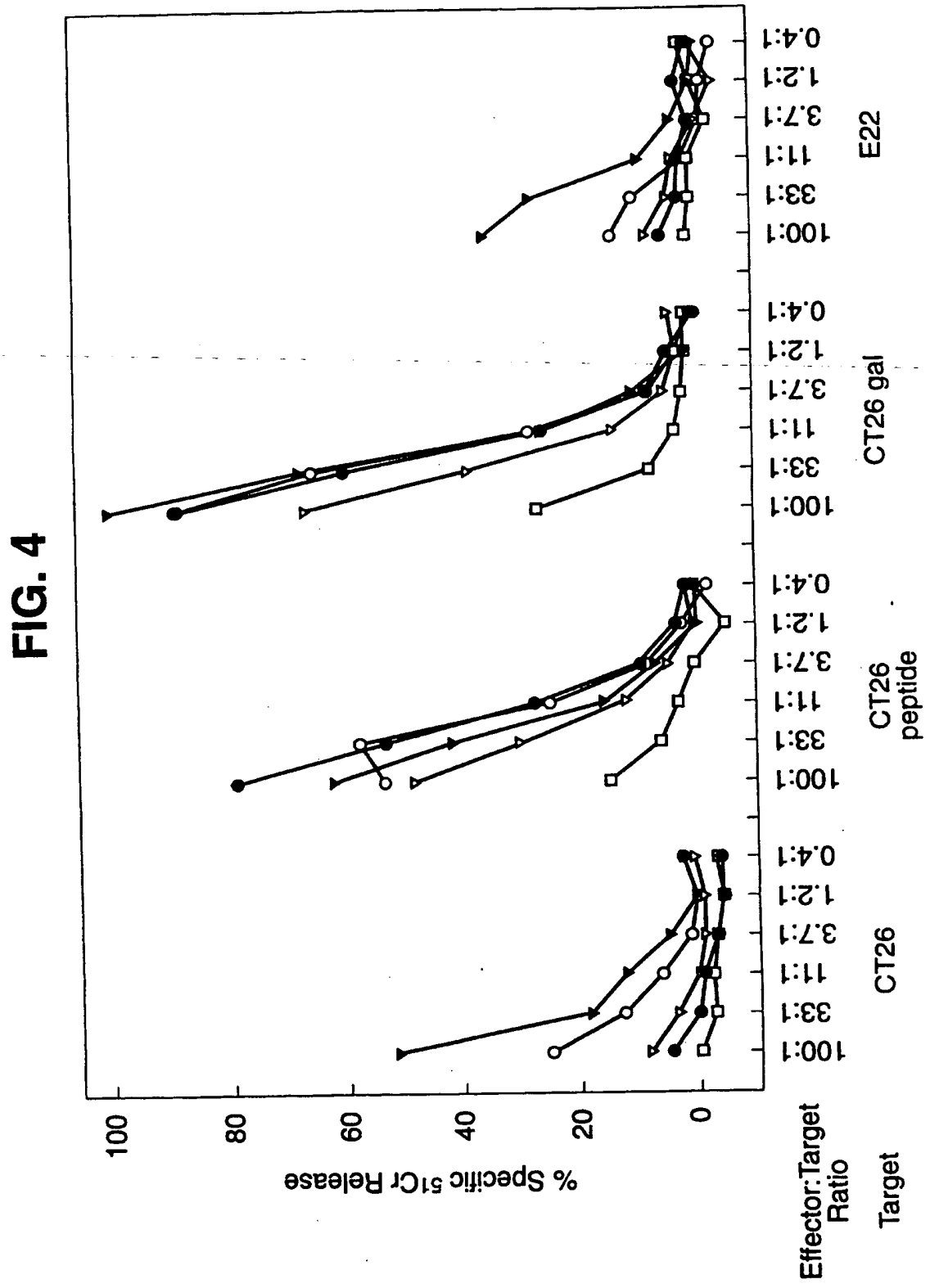

% Specific 51Cr Release
100
80
60
40
20
0
Effector:Target Ratio
Target
100:1
33:1
11:1
3.7:1
1.2:1
0.4:1
CT26
CT26 peptide
CT26 gal
E22

FIG. 6

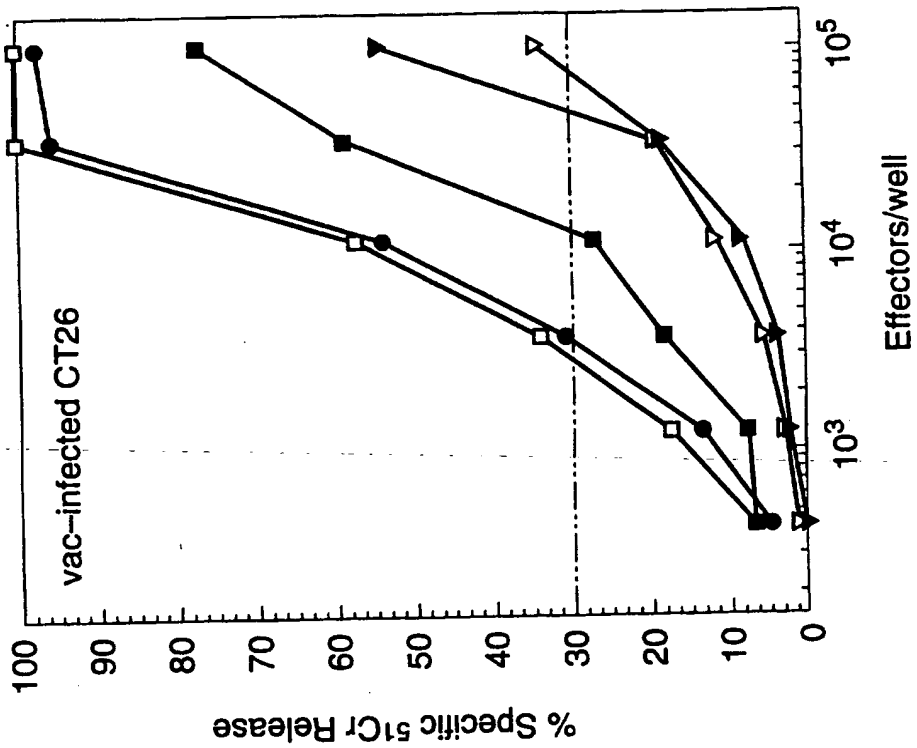

vac-infected CT26
% Specific 51Cr Release
100
90
80
70
60
50
40
30
20
10
0
Effectors/well
10^5
10^4
10^3

FIG. 5

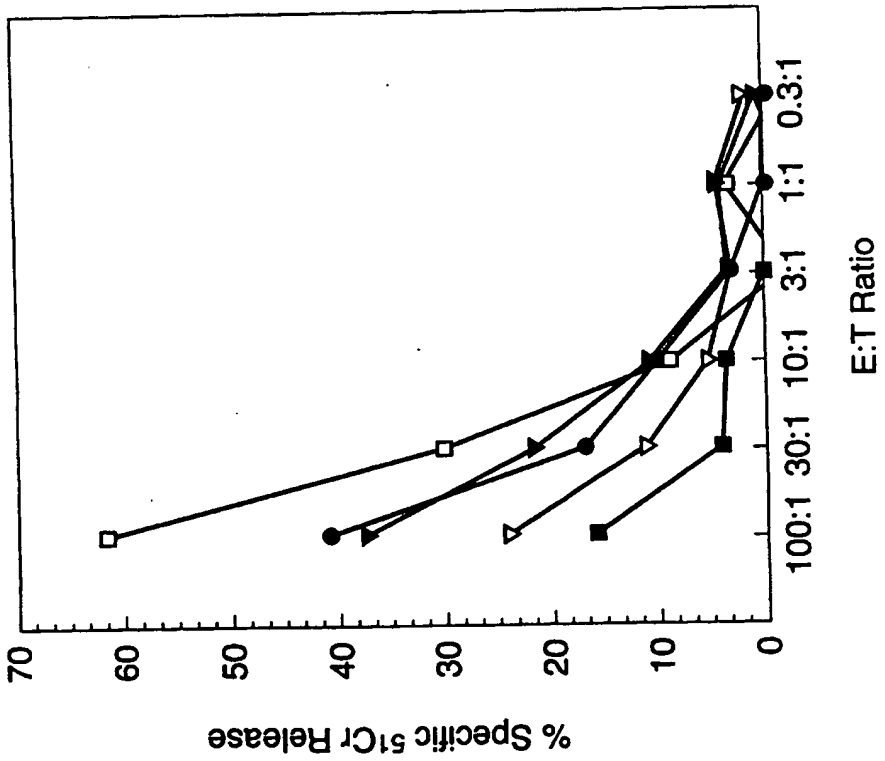

% Specific 51Cr Release
70
60
50
40
30
20
10
0
E:T Ratio
100:1
30:1
10:1
3:1
1:1
0.3:1

FIG. 7

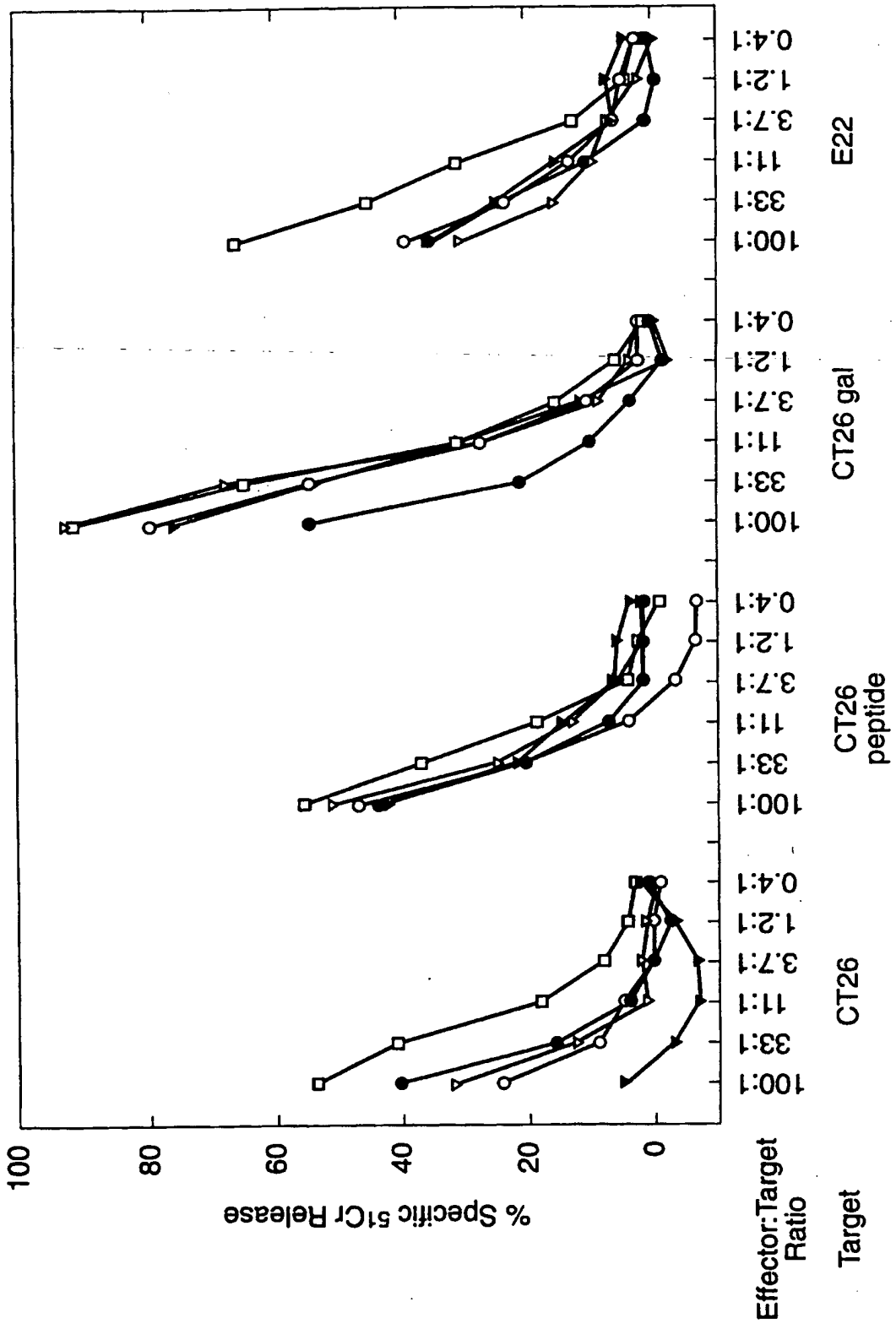

% Specific 51Cr Release
100
80
60
40
20
0
Effector:Target Ratio
Target
100:1
33:1
11:1
3.7:1
1.2:1
0.4:1
CT26
CT26 peptide
CT26 gal
E22

FIG. 8

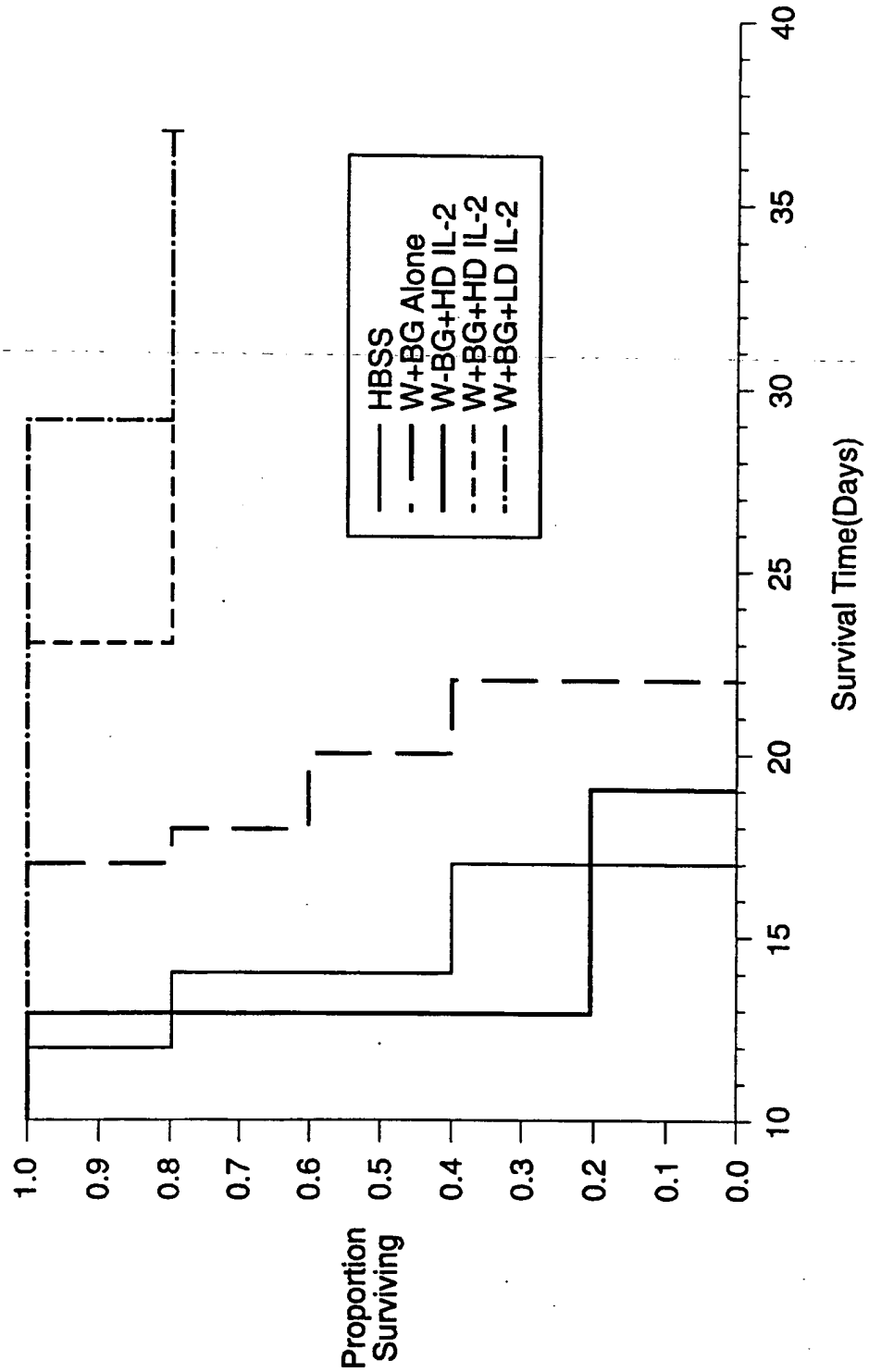

HBSS
W+BG Alone
W-BG+HD IL-2
W+BG+HD IL-2
W+BG+LD IL-2
Proportion Surviving
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
10
15
20
25
30
35
40
Survival Time(Days)

FIG. 9

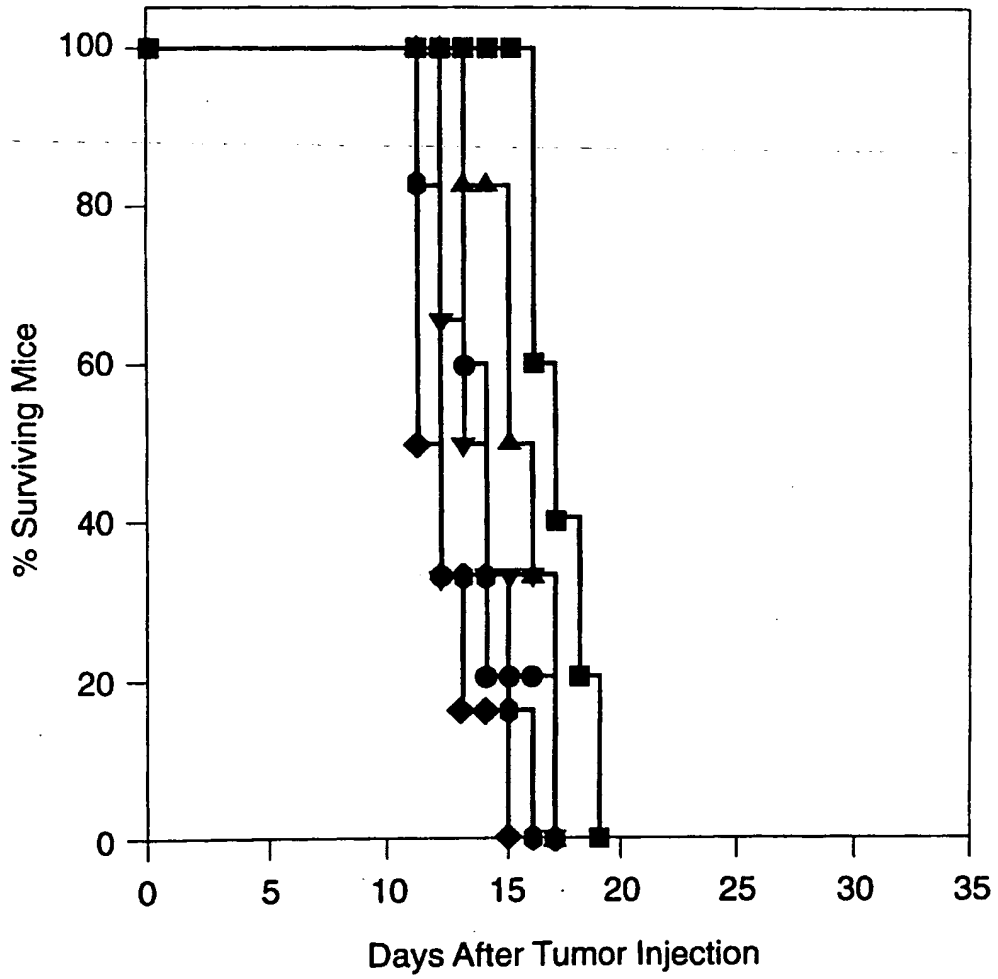

100
80
60
40
20
0
% Surviving Mice
0
5
10
15
20
25
30
35
Days After Tumor Injection

FIG. 10

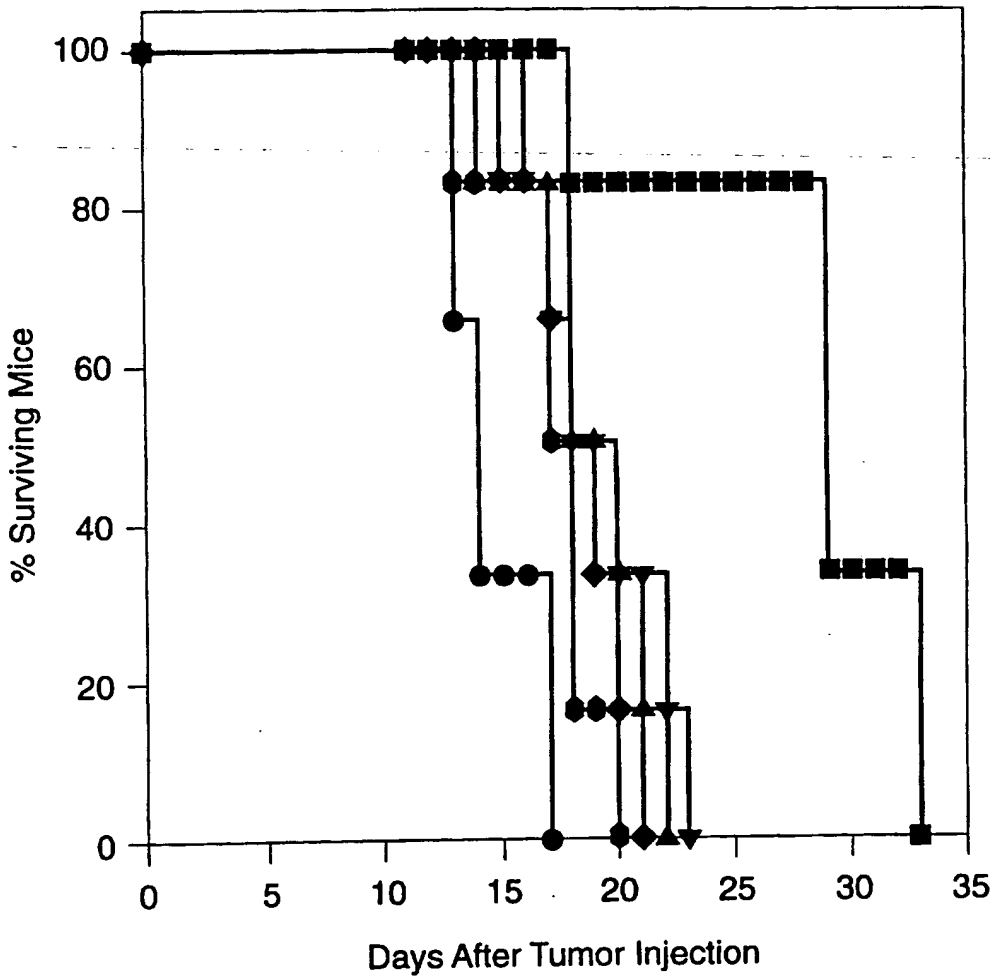

100
80
60
40
20
0
% Surviving Mice
0
5
10
15
20
25
30
35
Days After Tumor Injection

FIG. 11

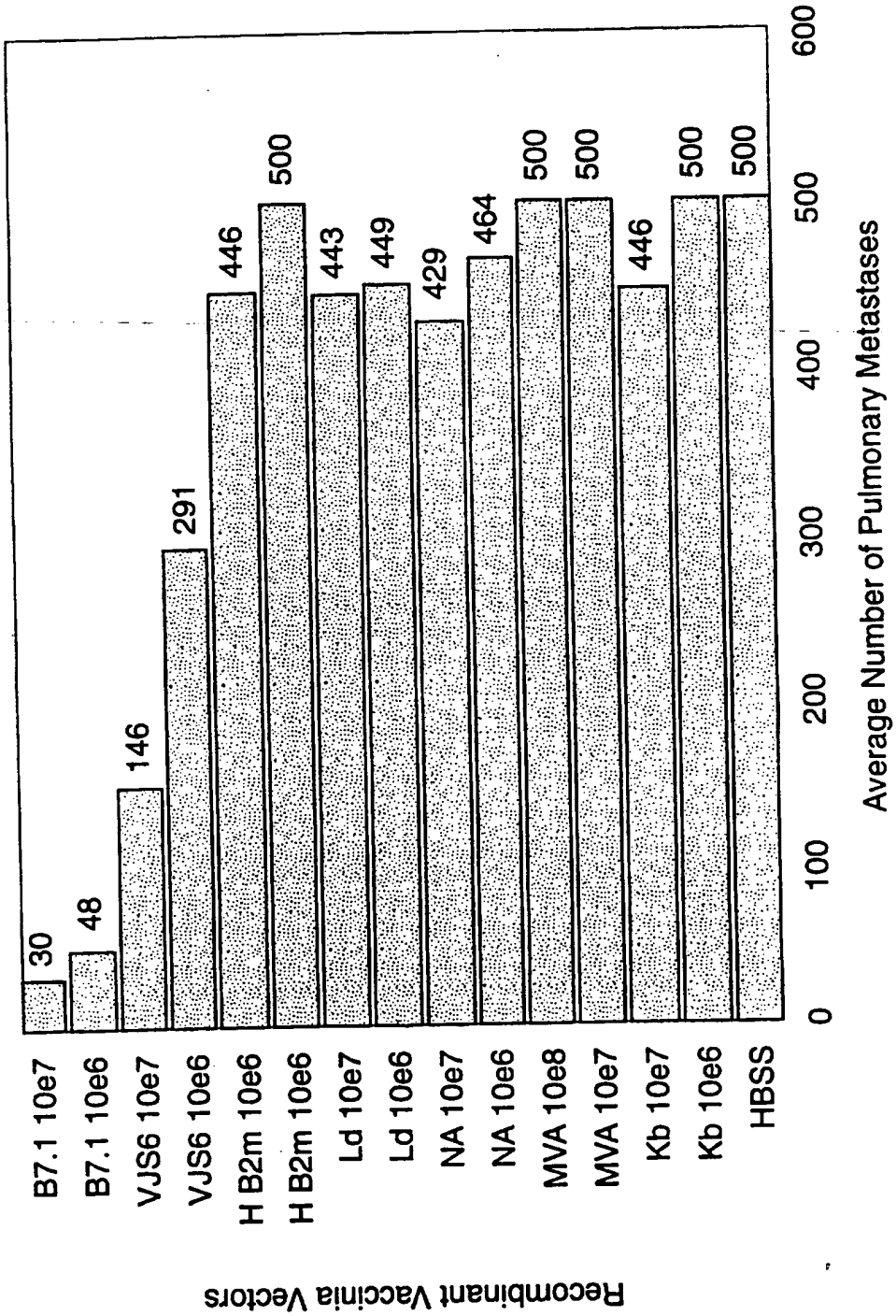

Average Number of Pulmonary Metastases
0
100
200
300
400
500
600
30
48
146
291
446
500
443
449
429
464
500
500
446
500
500
B7.1 10e7
B7.1 10e6
VJS6 10e7
VJS6 10e6
H B2m 10e6
H B2m 10e6
Ld 10e7
Ld 10e6
NA 10e7
NA 10e6
MVA 10e8
MVA 10e7
Kb 10e7
Kb 10e6
HBSS
Recombinant Vaccinia Vectors

FIG. 12

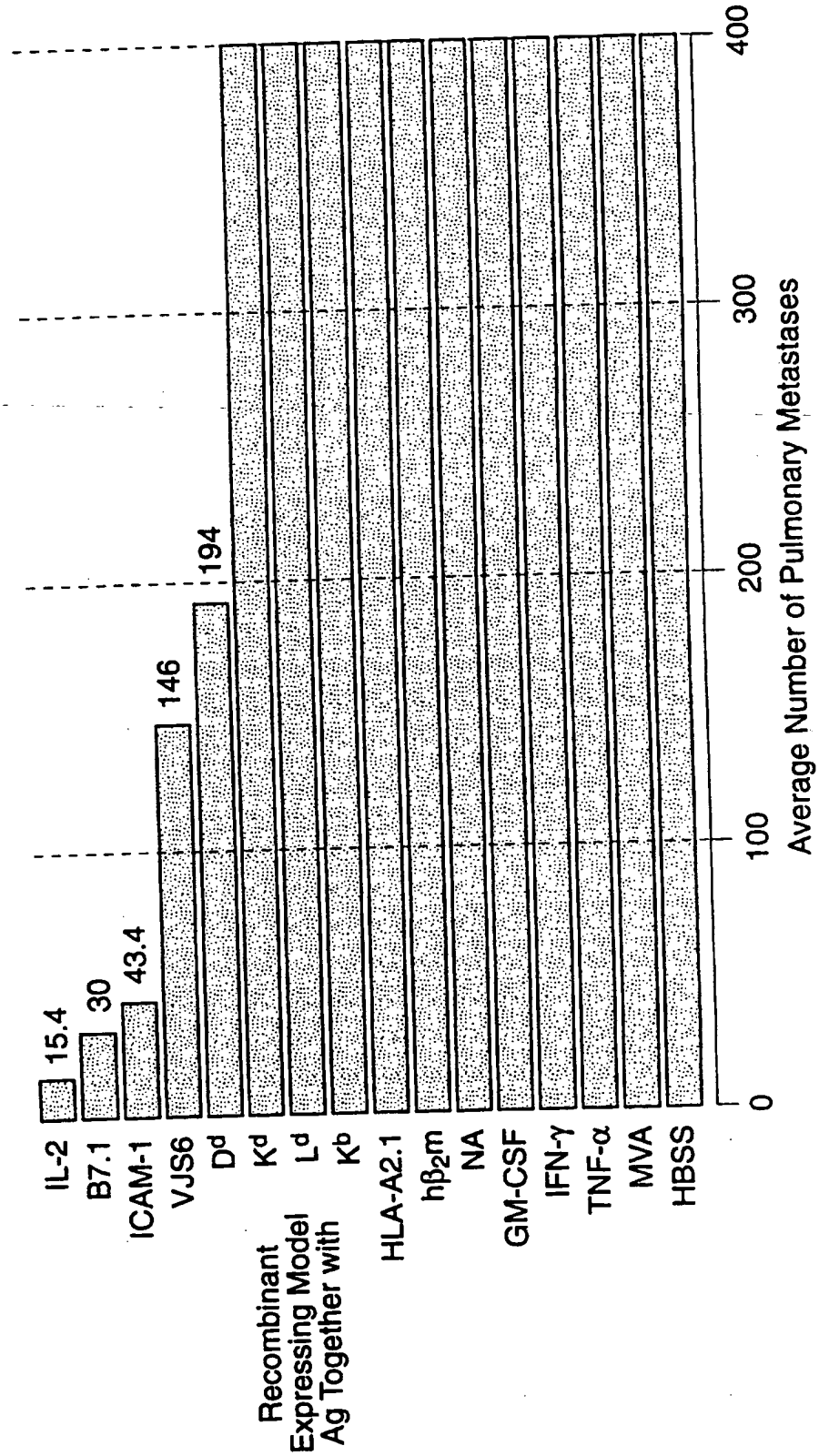

Average Number of Pulmonary Metastases
0
100
200
300
400
15.4
30
43.4
146
194
IL-2
B7.1
ICAM-1
VJS6
D^d
K^d
L^d
K^b
HLA-A2.1
$h\beta_2m$
NA
GM-CSF
IFN-γ
TNF-α
MVA
HBSS
Recombinant Expressing Model Ag Together with

FIG. 13

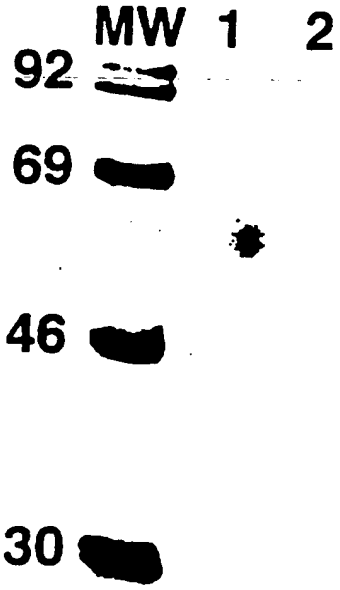


FIG. 14

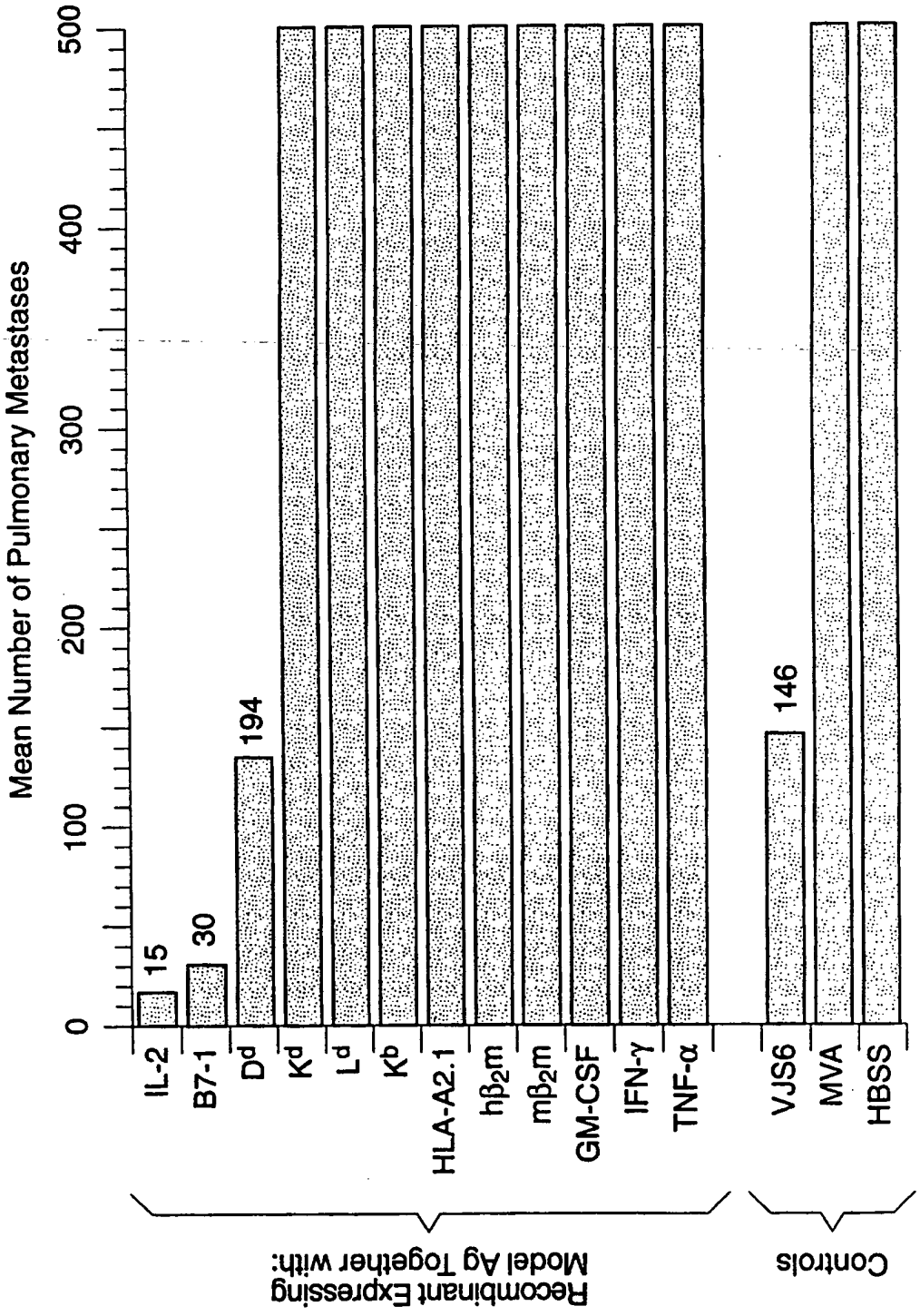

Mean Number of Pulmonary Metastases
0
100
200
300
400
500
15
30
194
146
IL-2
B7-1
D^d
K^d
L^d
K^b
HLA-A2.1
$h\beta_2m$
$m\beta_2m$
GM-CSF
IFN-γ
TNF-α
VJS6
MVA
HBSS
Recombinant Expressing Model Ag Together with:
Controls

FIG. 15

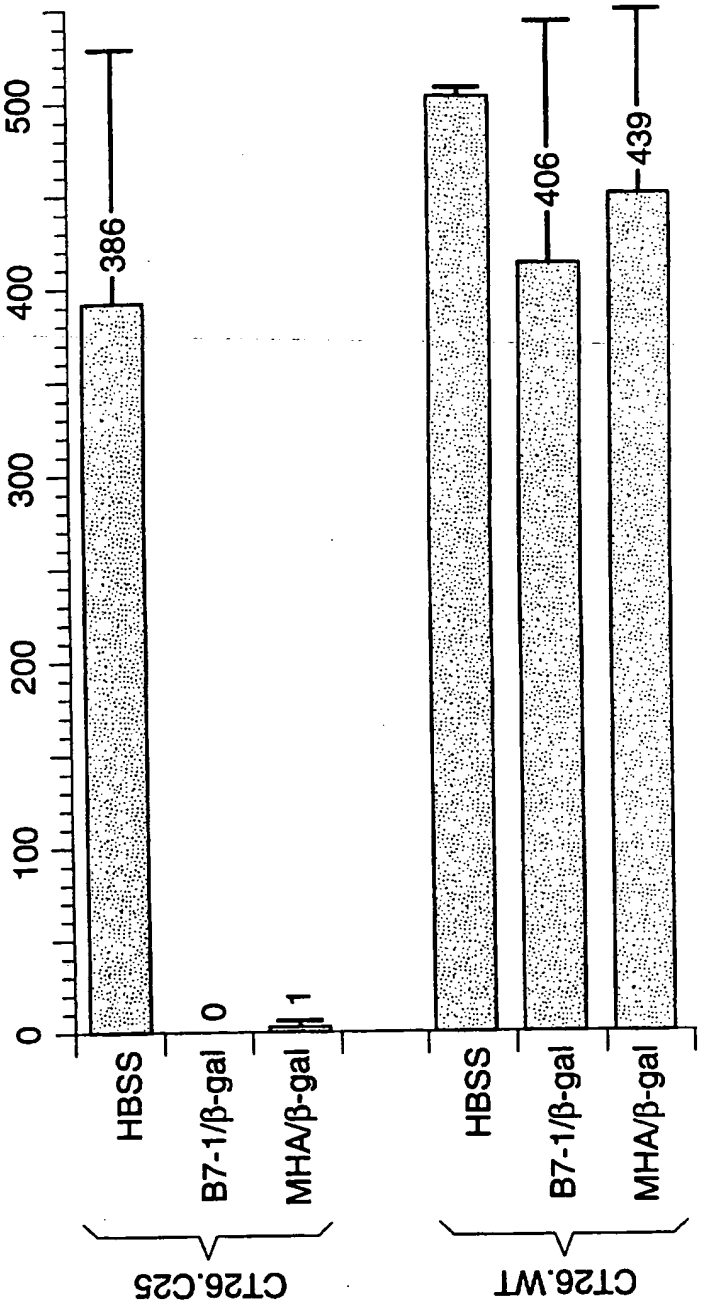

0
100
200
300
400
500
386
0
1
406
439
HBSS
B7-1/β-gal
MHA/β-gal
HBSS
B7-1/β-gal
MHA/β-gal
CT26.C25
CT26.WT

FIG. 16

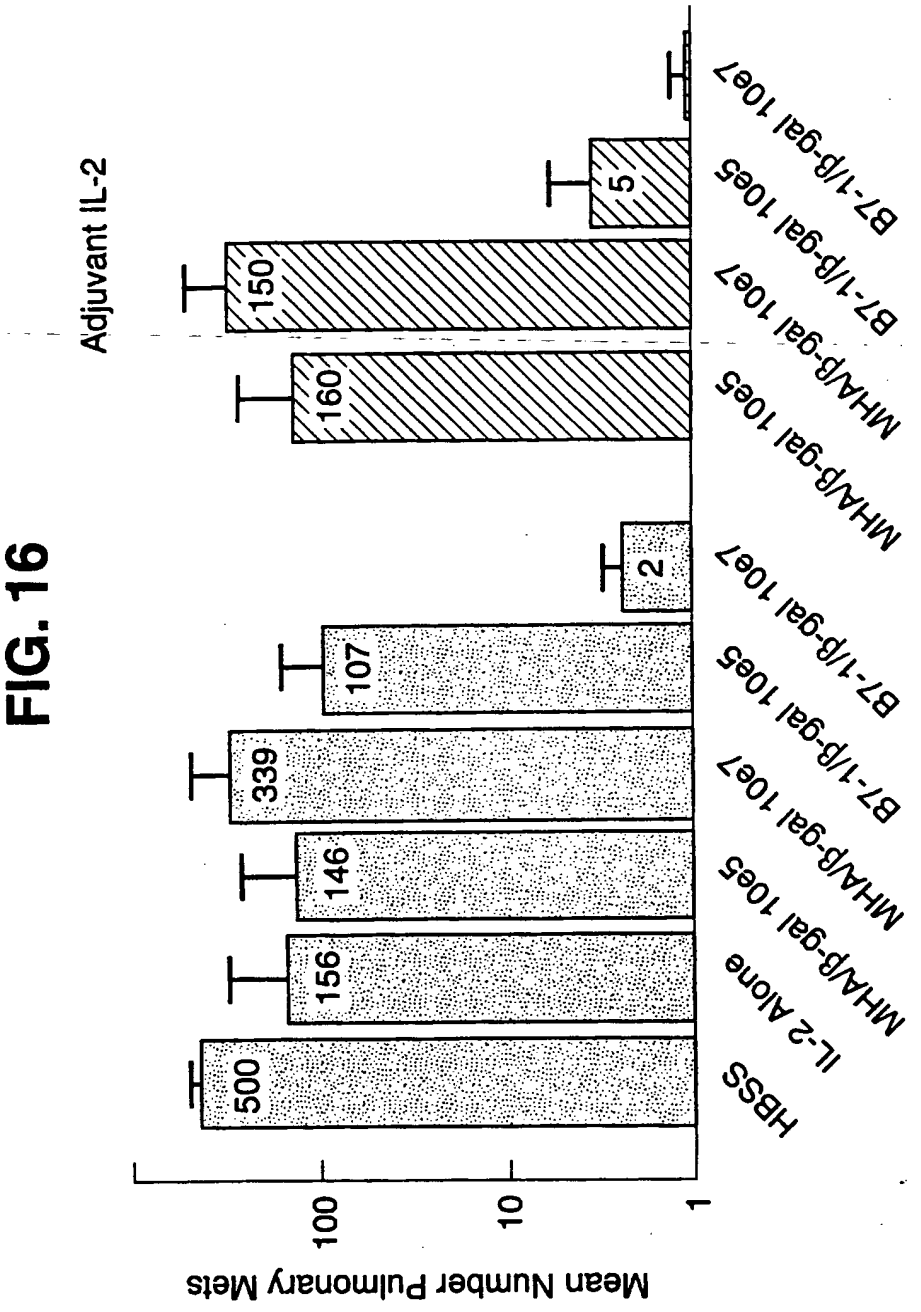

Adjuvant IL-2
Mean Number Pulmonary Mets
100
10
1
500
156
146
339
107
2
160
150
5
HBSS
IL-2 Alone
MHAβ-gal 10e5
MHAβ-gal 10e7
B7-1β-gal 10e5
B7-1β-gal 10e7
MHAβ-gal 10e5
MHAβ-gal 10e7
B7-1β-gal 10e5
B7-1β-gal 10e7

FIG. 17

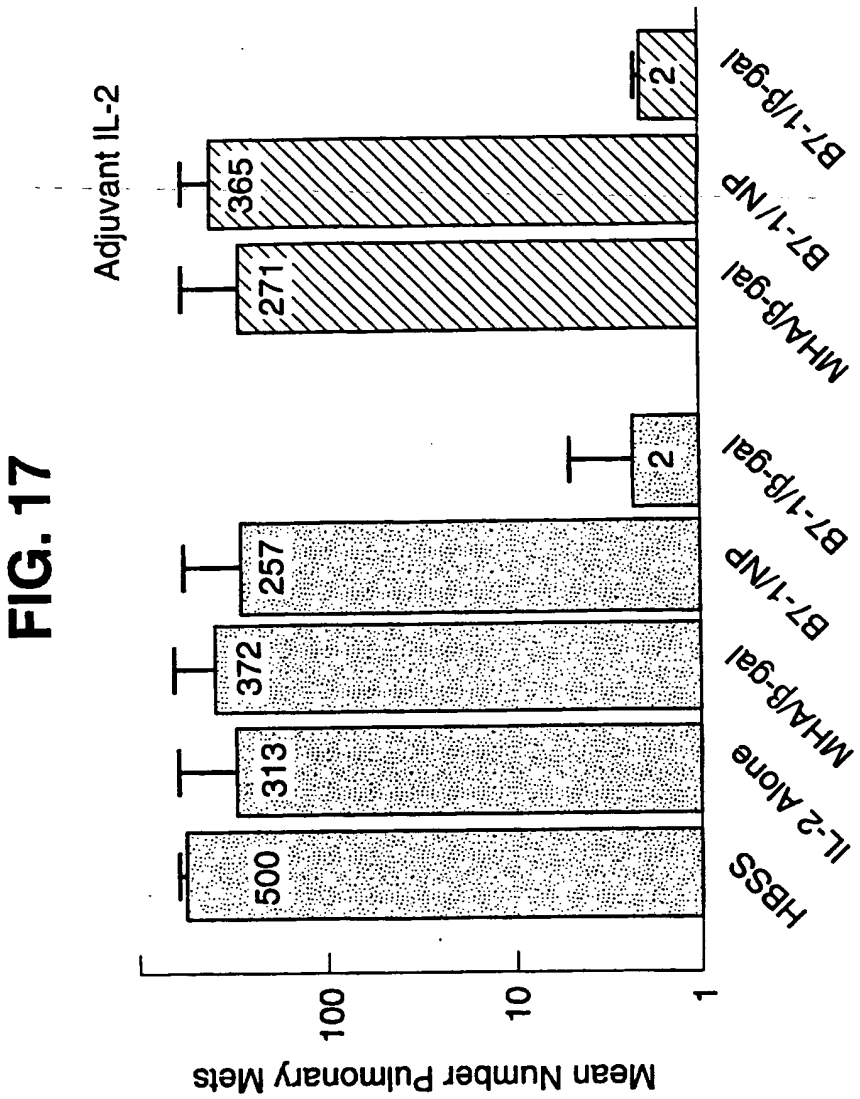

Adjuvant IL-2
Mean Number Pulmonary Mets
100
10
1
500
313
372
257
2
271
365
2
HBSS
IL-2 Alone
MHAβ-gal
B7-1/NP
B7-1/β-gal
MHAβ-gal
B7-1/NP
B7-1/β-gal

FIG. 18

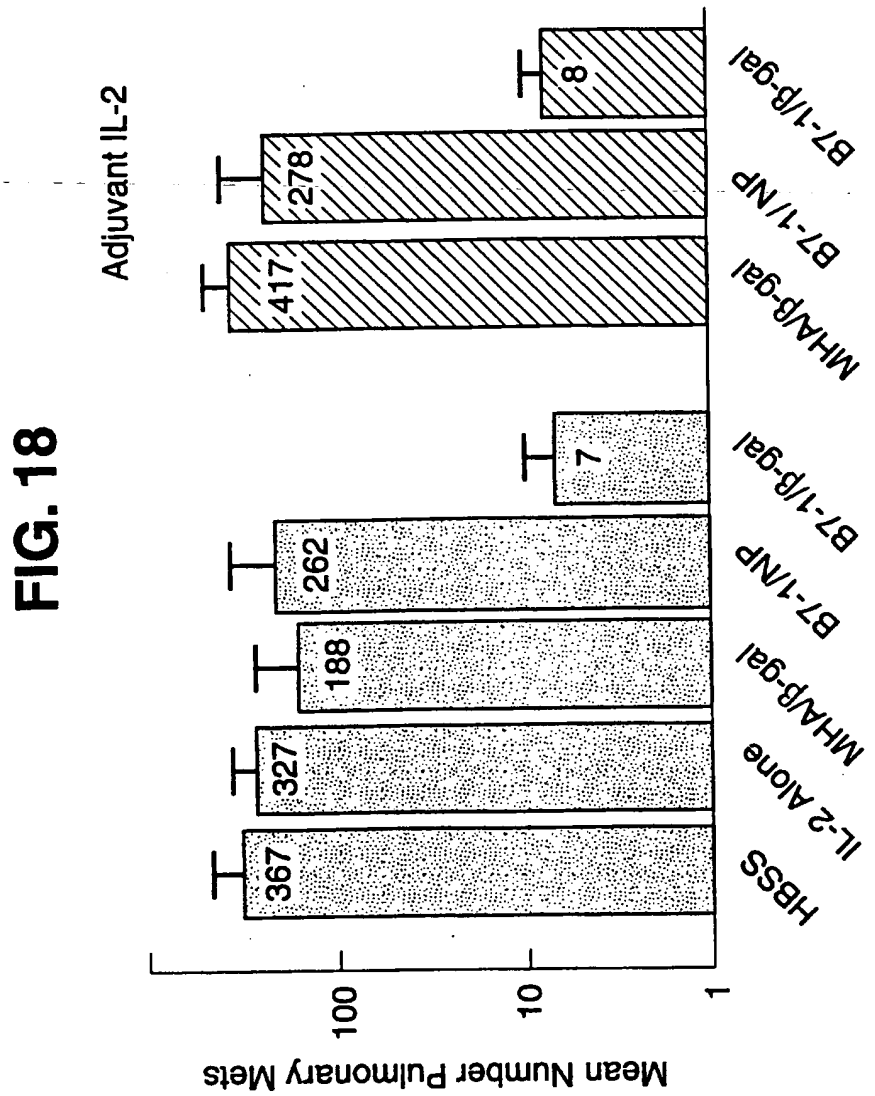

Mean Number Pulmonary Mets
100
10
1
HBSS
IL-2 Alone
MHAJβ-gal
B7-1/NP
B7-1/β-gal
MHAJβ-gal
B7-1/NP
B7-1/β-gal
Adjuvant IL-2
367
327
188
262
7
417
278
8

FIG. 19

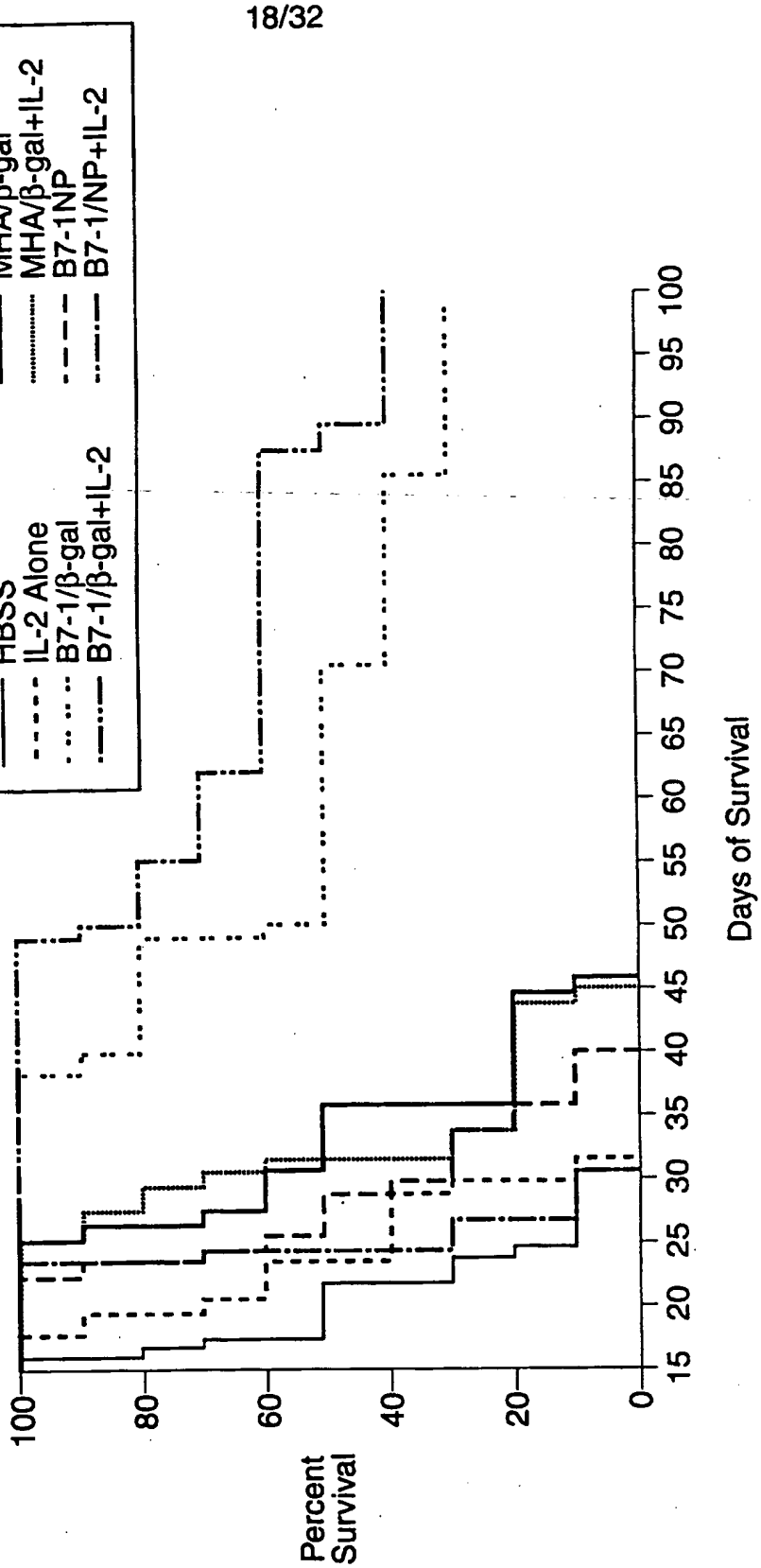

HBSS
IL-2 Alone
B7-1/β-gal
B7-1/β-gal+IL-2
MHA/β-gal
MHA/β-gal+IL-2
B7-1NP
B7-1/NP+IL-2
Percent Survival
100
80
60
40
20
0
15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100
Days of Survival

FIG. 20

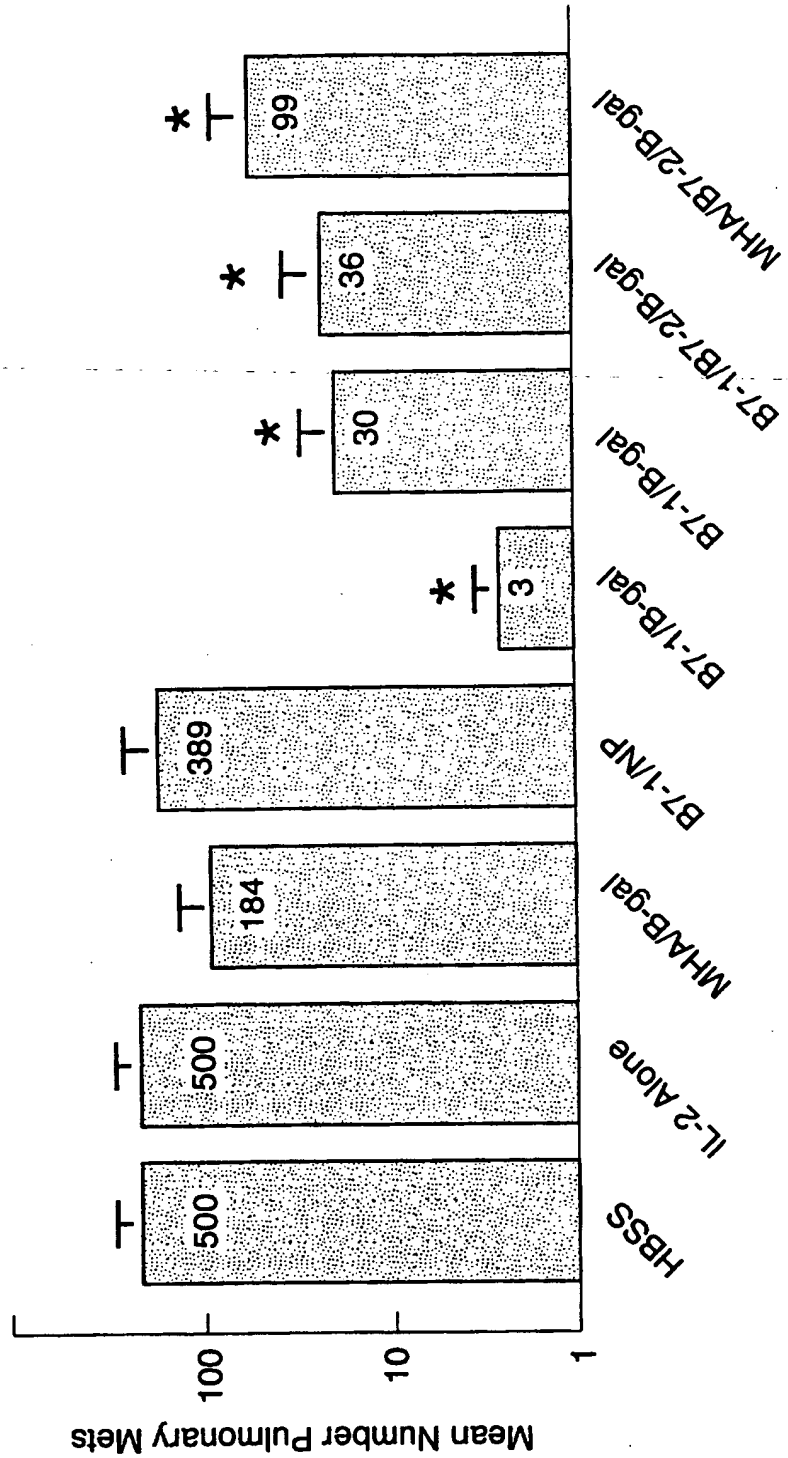

Mean Number Pulmonary Mets
100
10
1
HBSS
500
IL-2 Alone
500
MHA/B-gal
184
B7-1/NP
389
B7-1/B-gal
3
B7-1/B-gal
30
B7-1/B7-2/B-gal
36
MHA/B7-2/B-gal
99
*
*
*
*

FIG. 21a

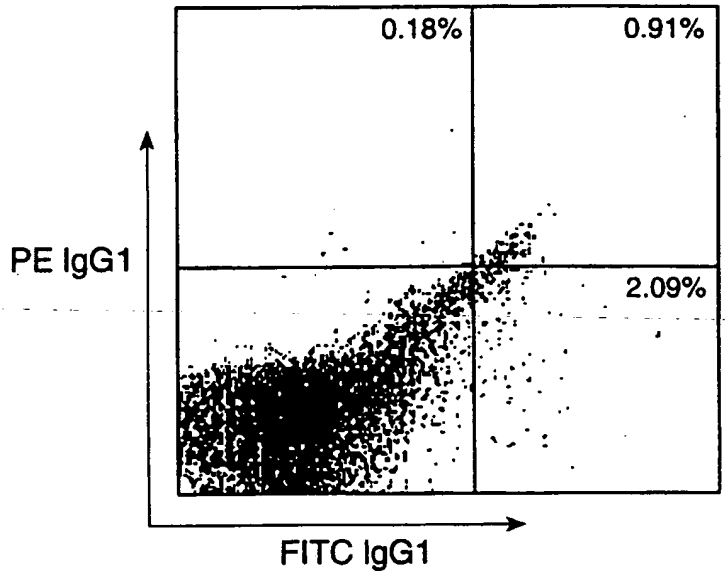


FIG. 21b

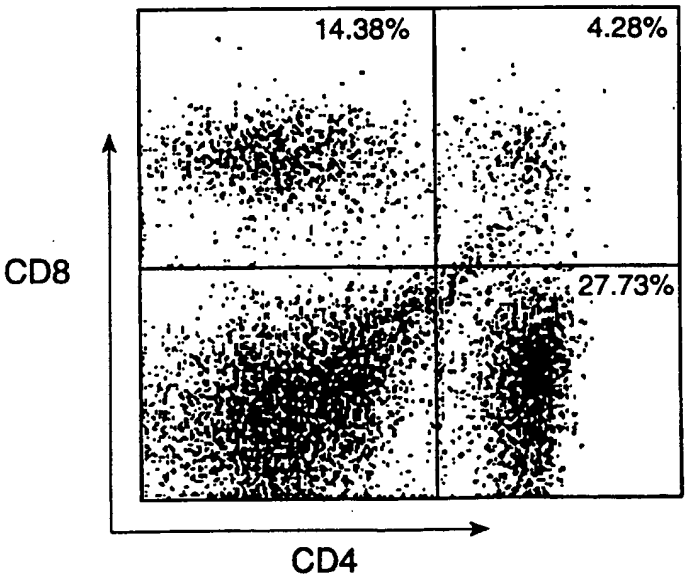


FIG. 21c

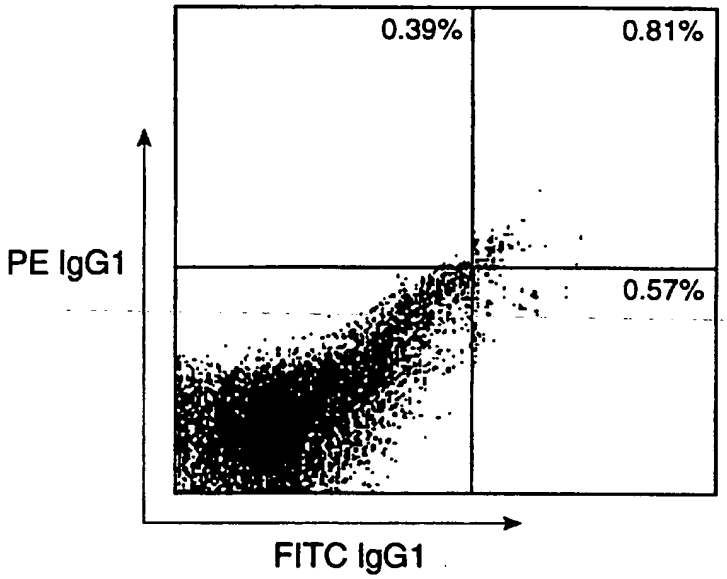


FIG. 21d

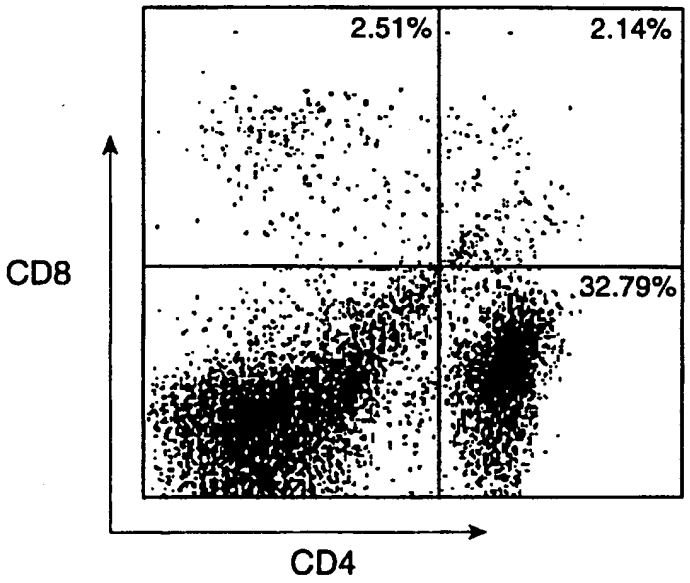


FIG. 21e

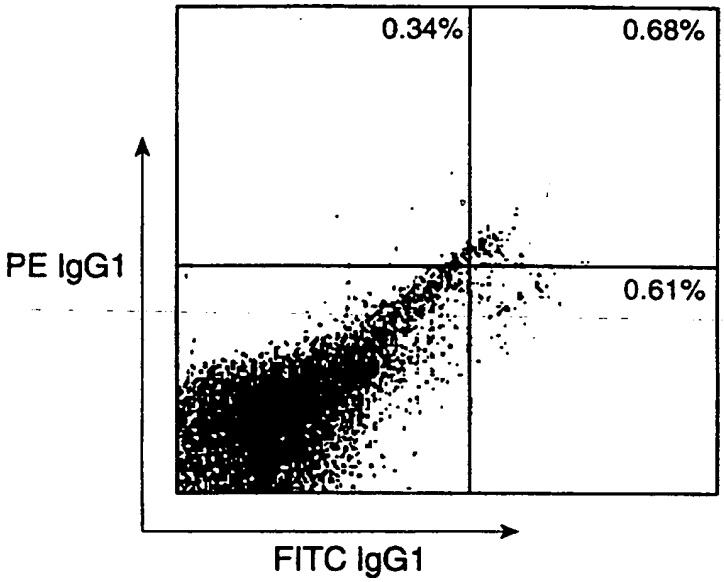


FIG. 21f

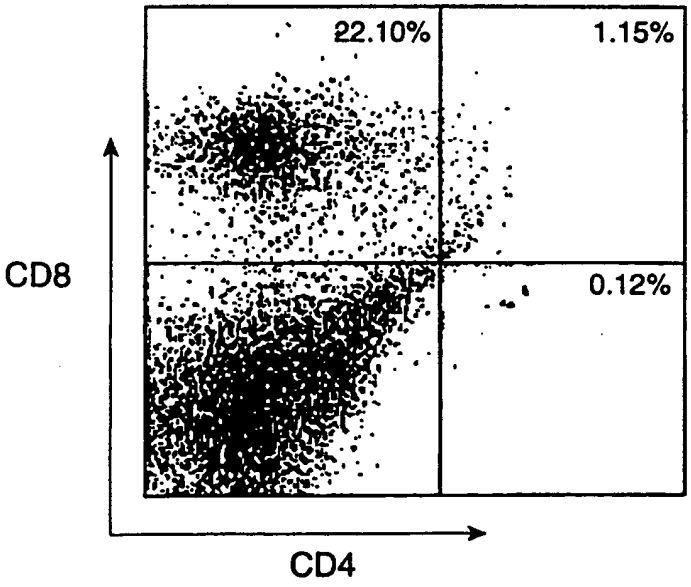


FIG. 21g

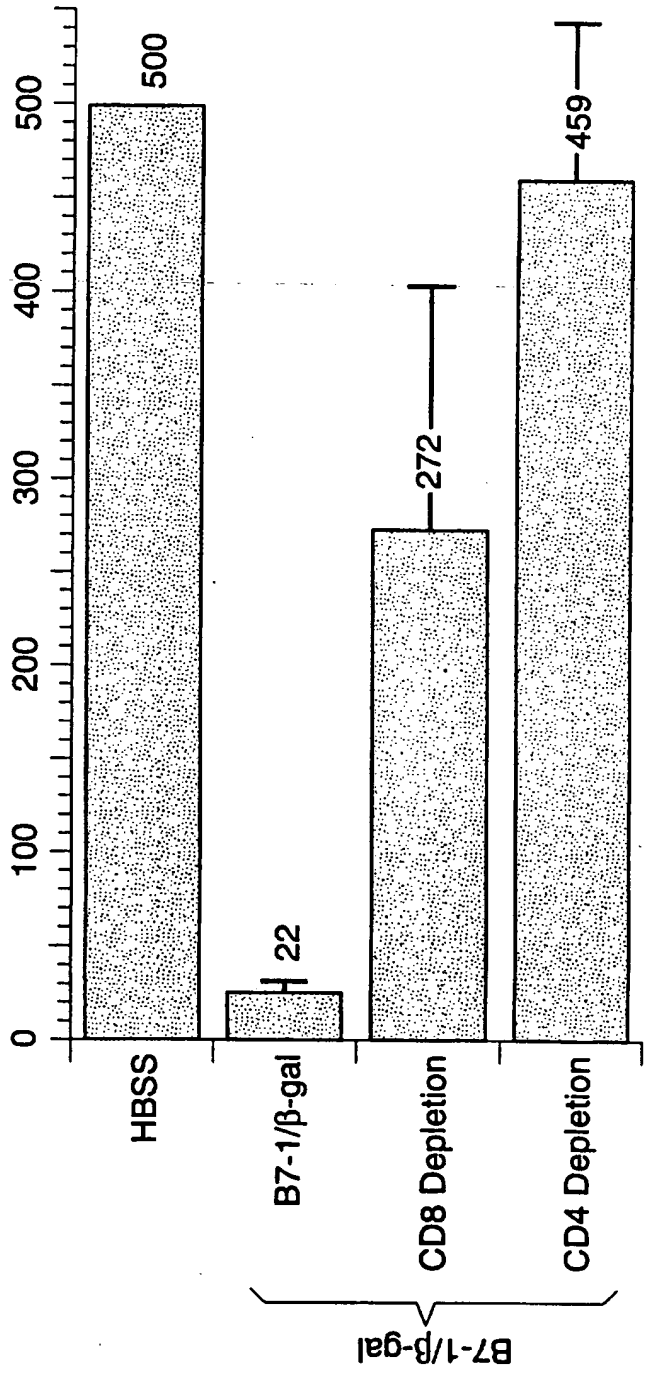

0
100
200
300
400
500
500
22
272
459
HBSS
B7-1/β-gal
CD8 Depletion
CD4 Depletion
B7-1/β-gal

FIG. 22a

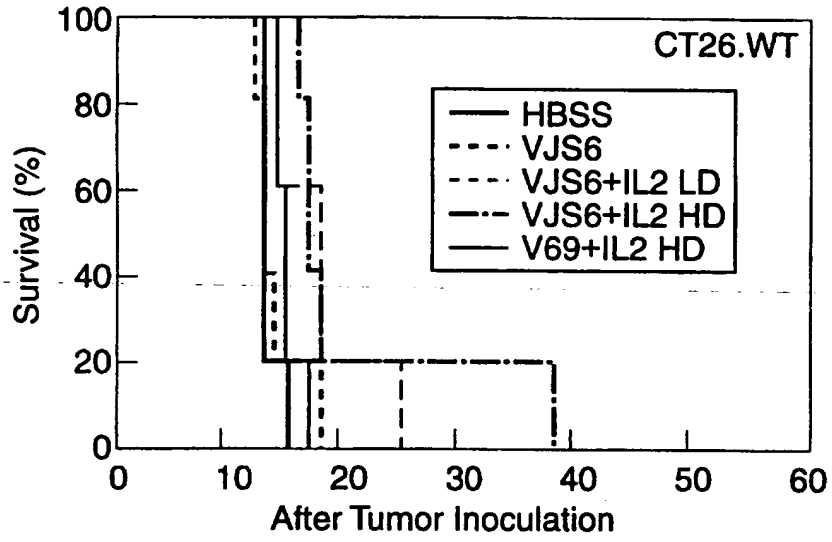


FIG. 22b

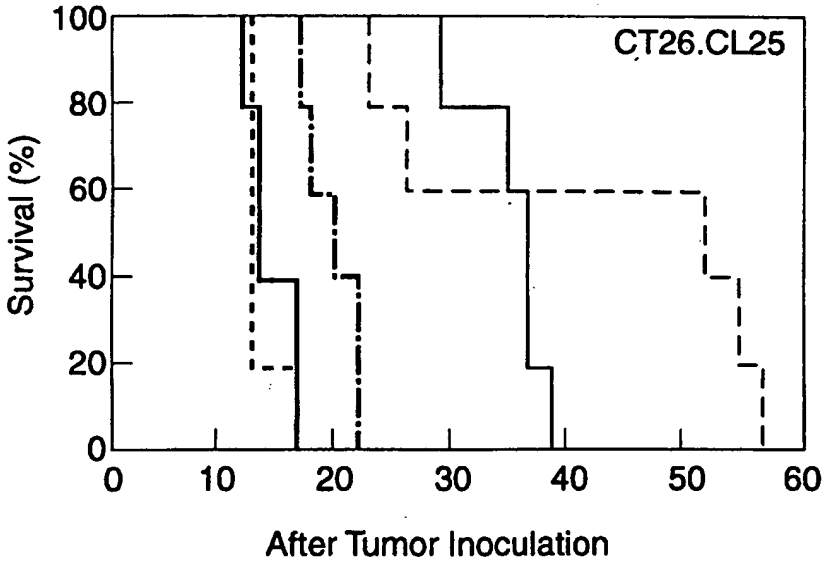


FIG. 23a

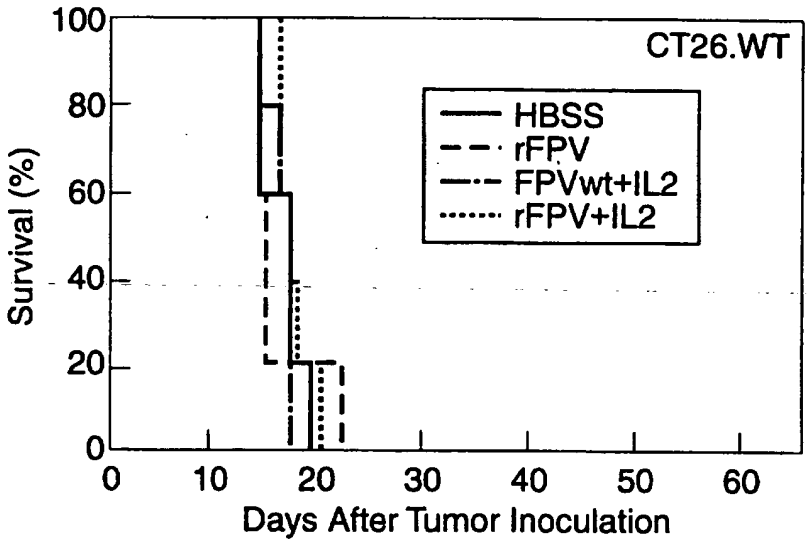


FIG. 23b

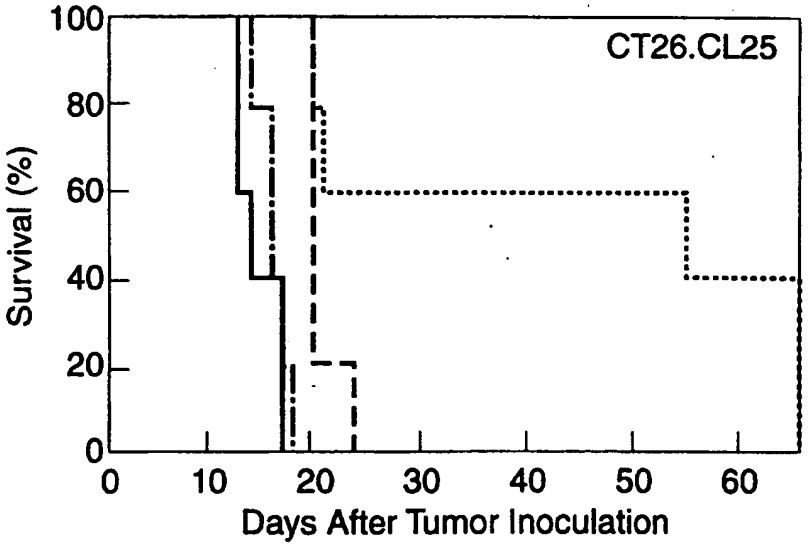


FIG. 24

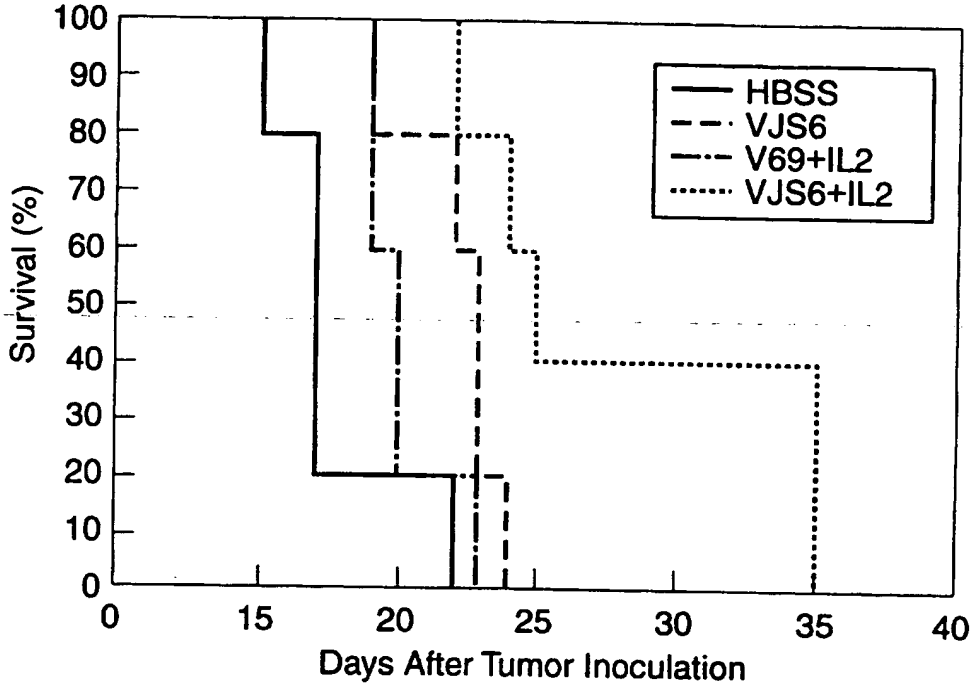


FIG. 25a

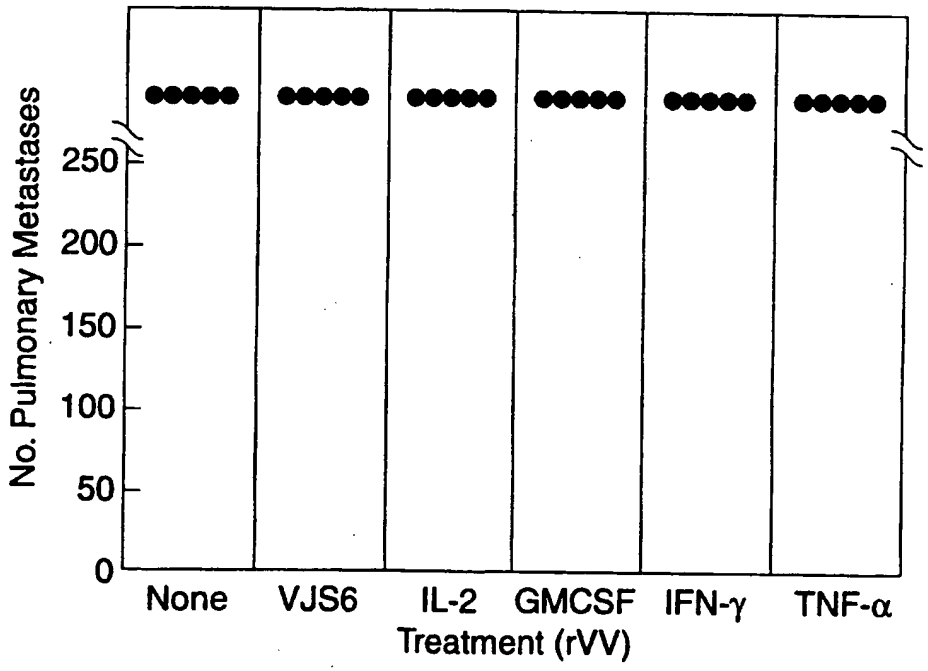


FIG. 25b

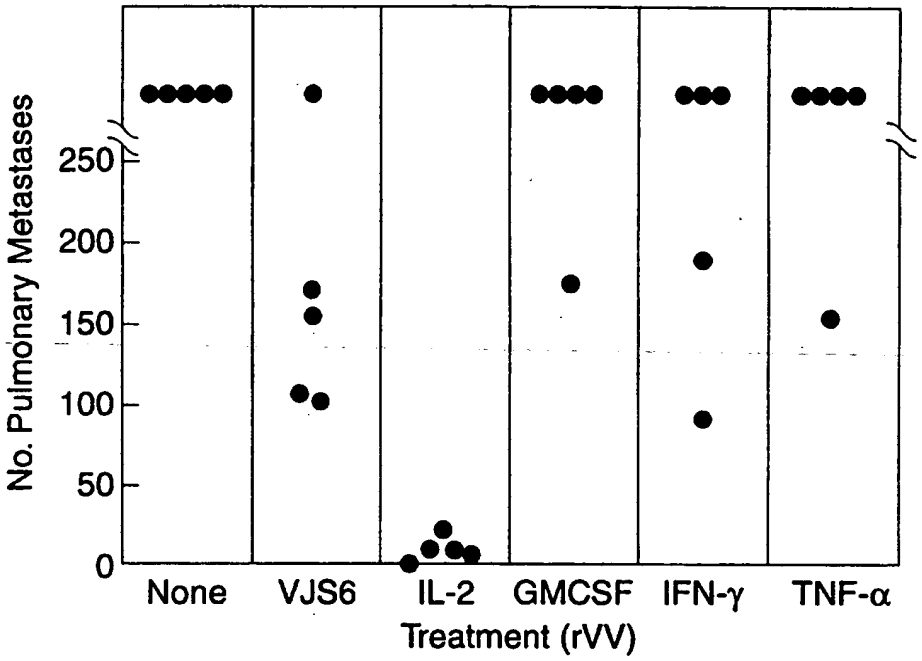


FIG. 26a

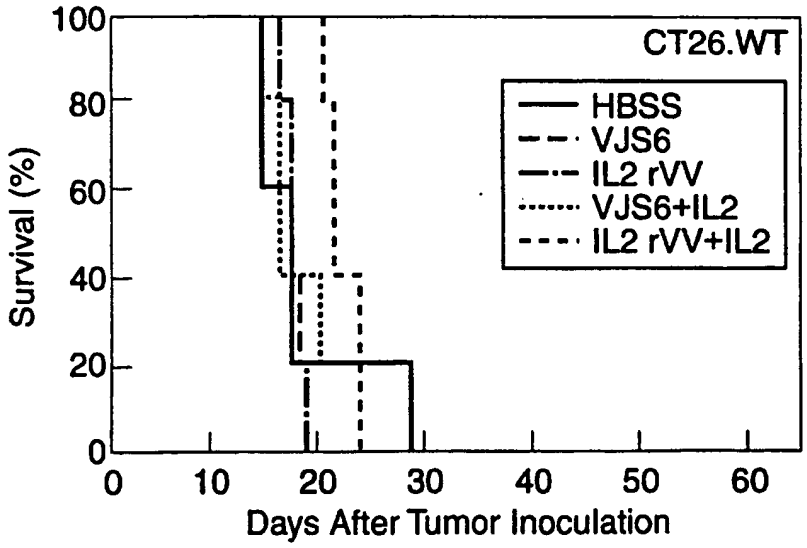


FIG. 26b

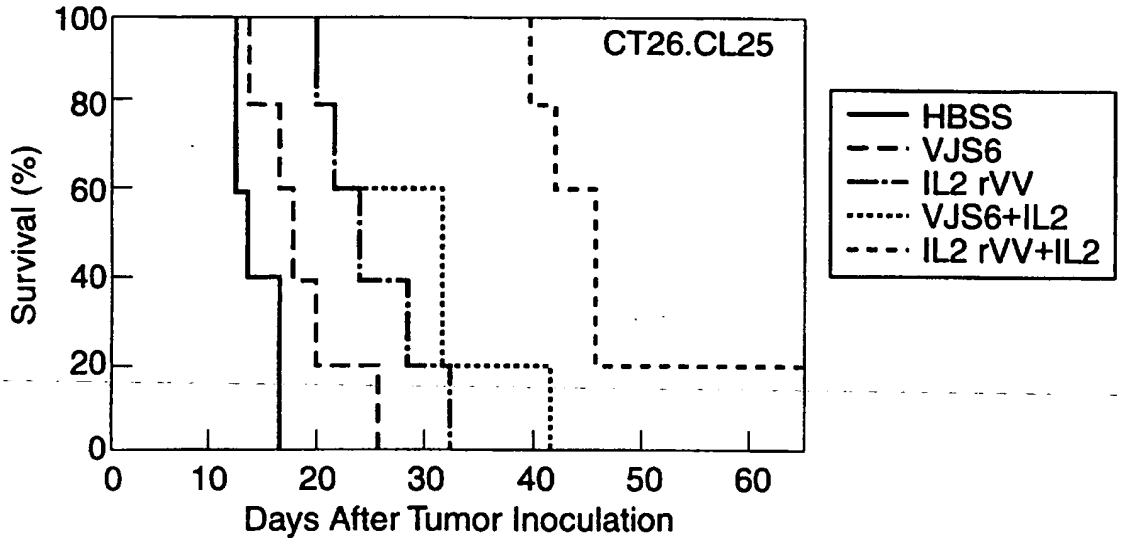


FIG. 27a

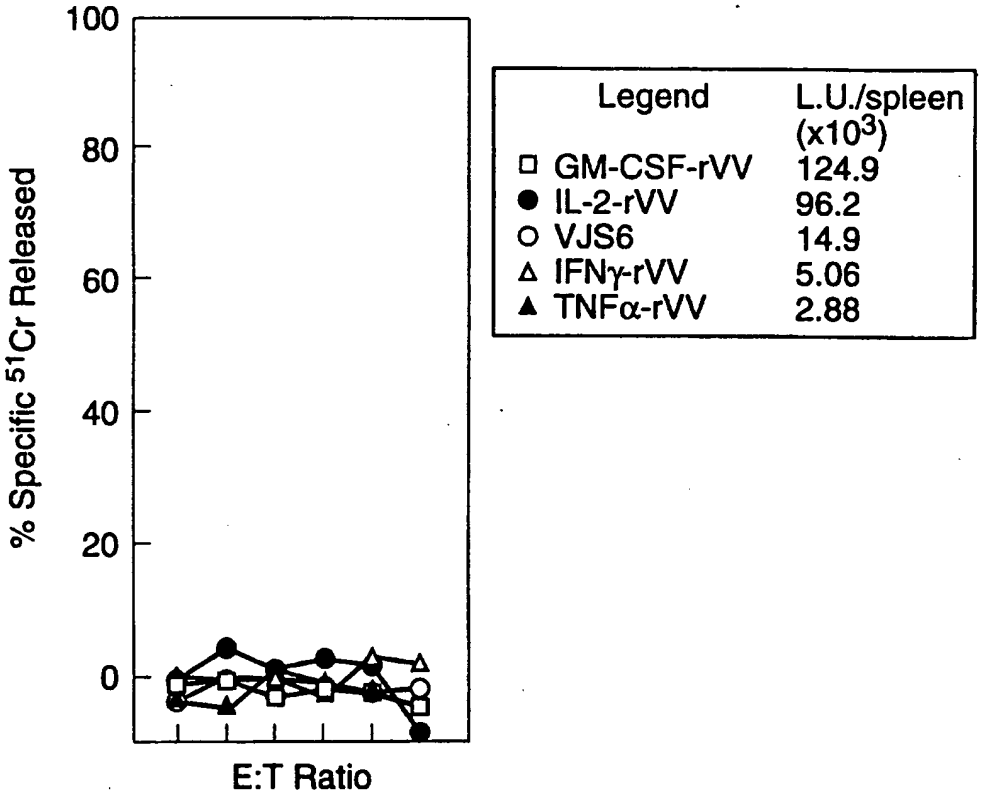


FIG. 28b

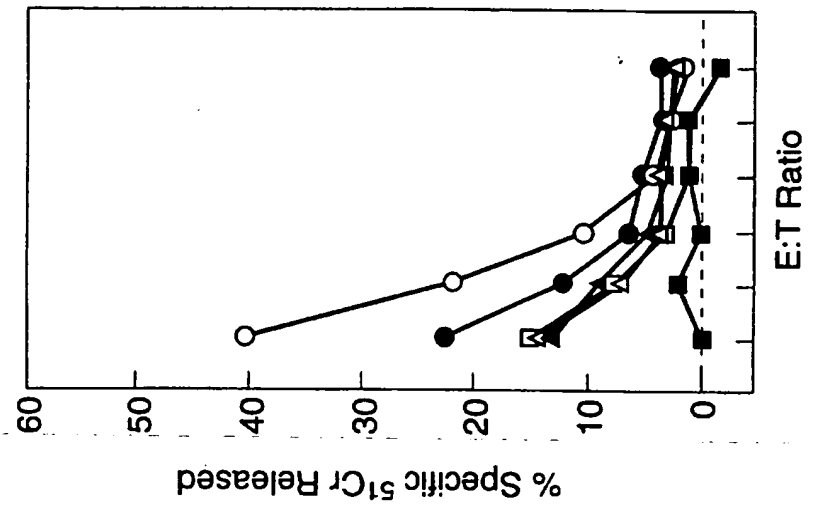


FIG. 28a

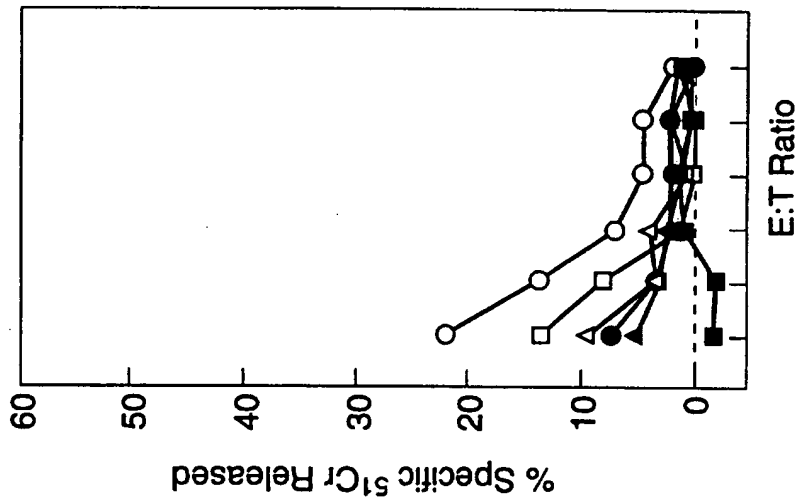


FIG. 27b

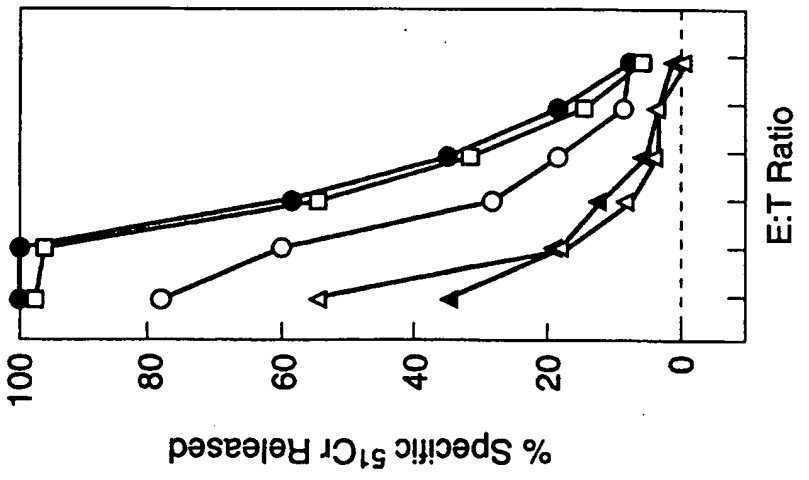


FIG. 28d

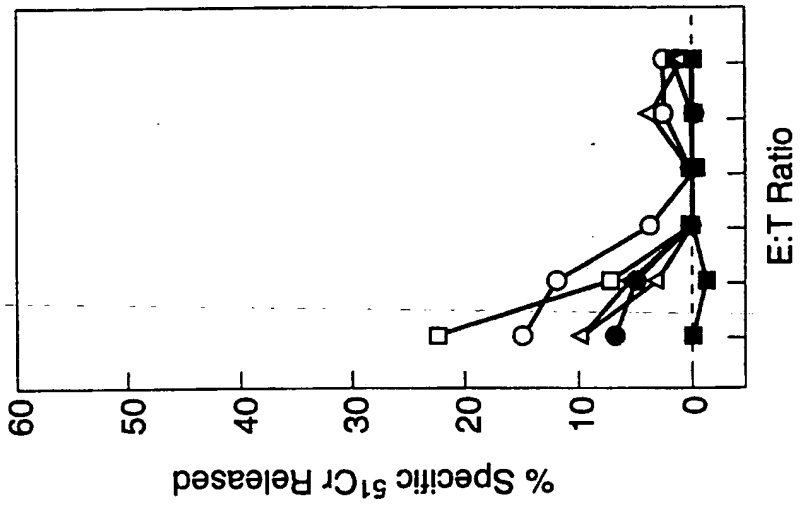


FIG. 28c

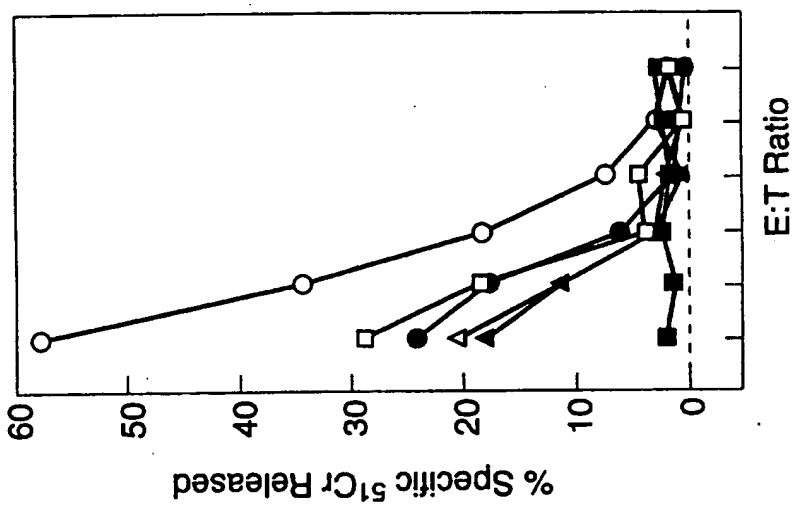


FIG. 29

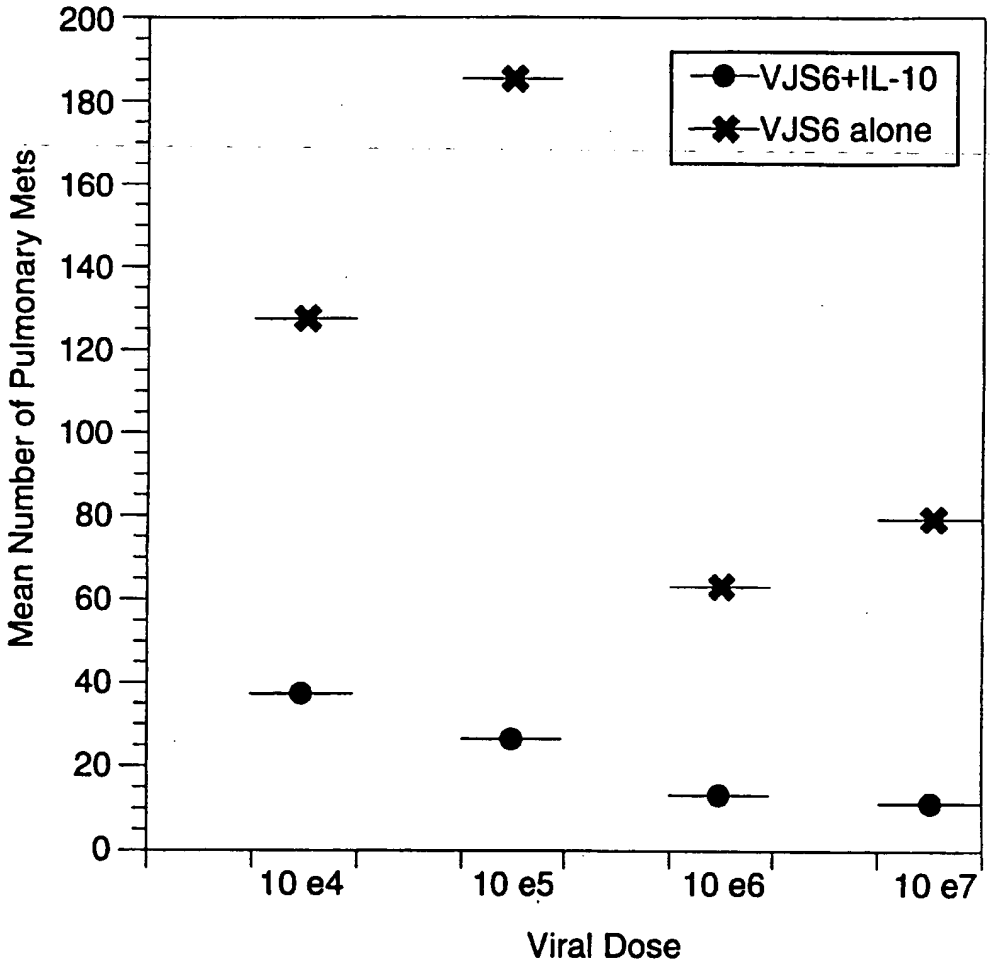
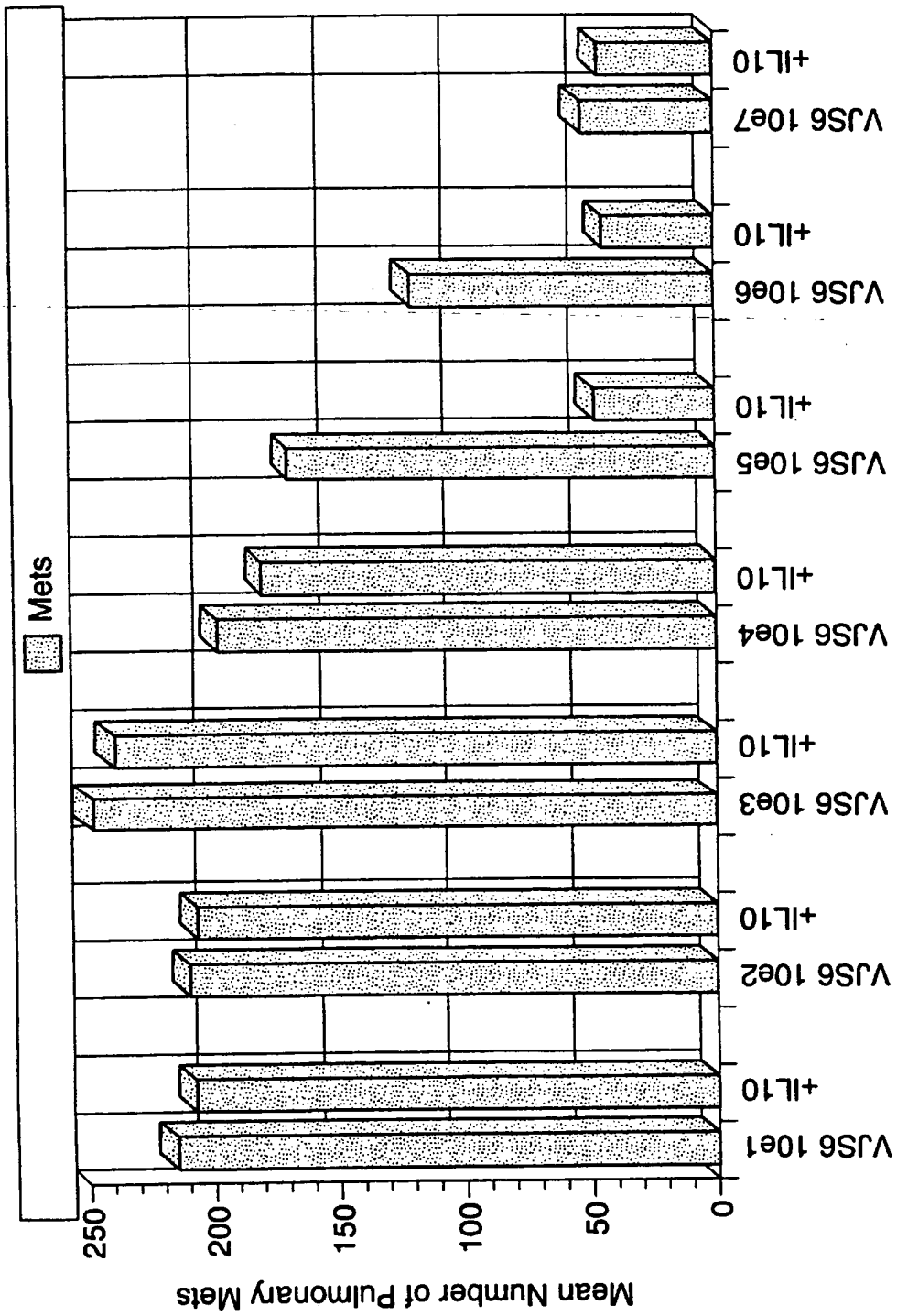

200
180
160
140
120
100
80
60
40
20
0
Mean Number of Pulmonary Mets
VJS6+IL-10
VJS6 alone
10 e4
10 e5
10 e6
10 e7
Viral Dose

FIG. 30


Mets
Mean Number of Pulmonary Mets
0
50
100
150
200
250
VJS6 10e1
+IL10
VJS6 10e2
+IL10
VJS6 10e3
+IL10
VJS6 10e4
+IL10
VJS6 10e5
+IL10
VJS6 10e6
+IL10
VJS6 10e7
+IL10